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Autoantibodies to Brain Components and Antibodies to *Acinetobacter calcoaceticus* Are Present in Bovine Spongiform Encephalopathy

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▸ ABSTRACT

▲ Top
· Abstract
▼ Introduction
▼ Materials and Methods
▼ Results
▼ Discussion
▼ References

Bovine spongiform encephalopathy (BSE) is a neurological disorder, predominantly of British cattle, which belongs to the group of transmissible spongiform encephalopathies together with Creutzfeldt-Jakob disease (CJD), kuru, and scrapie. Autoantibodies to brain neurofilaments have been previously described in patients with CJD and kuru and in sheep affected by scrapie. Spongiform-like changes have also been observed in chronic experimental allergic encephalomyelitis, at least in rabbits and guinea pigs, and in these conditions autoantibodies to myelin occur. We report here that animals with BSE have elevated levels of immunoglobulin A autoantibodies to brain components, i.e., neurofilaments ($P < 0.001$) and myelin ($P < 0.001$), as well as to *Acinetobacter calcoaceticus* ($P < 0.001$), saprophytic microbes found in soil which have sequences cross-reacting with bovine neurofilaments and myelin, but there were no antibody elevations against *Agrobacterium tumefaciens* or *Escherichia coli*. The relevance of such mucosal autoantibodies or antibacterial antibodies to the pathology of BSE and its possible link to prions requires further evaluation.

▸ INTRODUCTION

▲ Top
▲ Abstract
• Introduction
▼ Materials and Methods
▼ Results
▼ Discussion
▼ References

Bovine spongiform encephalopathy (BSE) is a recently discovered neurological disorder of cattle which was first reported in the United Kingdom after 1985, following a change in the preparation of "meat and bone meal" (MBM) feeds used especially during the winter months (1). The disorder has attracted public concern lest it be transmitted to humans following consumption of meat or other animal products (20). It has been suggested that BSE is caused by either abnormal prions (PrP^{sc}) (11, 12) or exposure to organophosphates (13) and belongs to the group of transmissible spongiform encephalopathies (TSEs) together with kuru, Creutzfeldt-Jakob disease, and scrapie, conditions in which autoantibodies to brain neurofilaments have been described by Gajdusek's group (2, 15).

A characteristic histopathological feature of BSE is a spongiform appearance, which also occurs in chronic but not acute experimental allergic encephalomyelitis (10, 14), a condition in which autoantibodies to myelin occur (17), but its possible link to BSE has so far not been examined. A short sequence of bovine myelin (RFSWGAEGQK) resistant to denaturation by heating to 100°C for 1 h or by treatment with 8 M urea (a resistance which it shares with prion molecules) was reported over 25 years ago to produce ataxia, hind quarter paralysis, tremors, and eventually death following inoculation into guinea pigs (5). These features resemble, to some extent, those observed in cattle affected by BSE. This sequence was used as a computer probe to search protein databases for bacterial and viral proteins which may show molecular mimicry to bovine brain tissues. Analysis of proteins in databases (GenBank and SwissProt) revealed that three microbes showed molecular mimicry with brain tissues, the best one being found in 4-carboxy-muconolactone-decarboxylase of *Acinetobacter calcoaceticus* (4), a common saprophytic microbe found in soil and water supplies (19) which also possesses sequences resembling bovine neurofilaments (Table 1). Furthermore, another common environmental microbe, *Agrobacterium tumefaciens*, also showed some similarities to bovine myelin, although not to the same extent as *A. calcoaceticus*. Further probing with published prion sequences (7) revealed similarities with three molecules (recognition protein, colicin M, and maltodextrin-glucosidase), all of which are found in *Escherichia coli* (4).

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TABLE 1. Comparison of similar sequences in bovine neurofilaments compared with *A. calcoaceticus* molecular sequences

BSE-affected cattle and healthy controls have been tested by enzyme-linked immunosorbent assay (ELISA) for the presence of autoantibodies to bovine neurofilaments and myelin and to these three common microorganisms (18). Since BSE was thought to be caused by consumption of MBM winter feeds, it was believed that the mucosal immunoglobulin A (IgA) isotype was more likely to show any possible differences in the titer of autoantibodies to brain components. Molecular modelling suggested three possible microbes which showed cross-reactivity, and these were tested by using a total Ig (IgG + IgA + IgM) assay in an endeavor to detect any immunological signal.

MATERIALS AND METHODS

Top
Abstract
Introduction
Materials and Methods
Results
Discussion
References

Sera from animals with or without BSE. Sera from 29 animals (mean age, 74.4 months; range, 44 to 122 months) which were found at postmortem to satisfy the criteria of having BSE and 18 animals which did not have the disorder were supplied by the Central Veterinary Laboratory (CVL) (New Haw, Addlestone, Surrey, England), an executive agency of the Ministry of Agriculture, Fisheries and Food. The 18 animals which did not have BSE had been referred to CVL because of abnormal behavior involving ataxia and suggesting a neurological disease. Postmortem examinations were carried out to exclude BSE. The BSE and control sera (CVL) were obtained from animals raised on farms in different parts of England, each having its own water supply and belonging to separate herds. The majority of the BSE-positive animals came from dairy Friesian herds. Specifically, there was no genetic or breeder link between the various animals that had developed BSE or the controls.

Sera from animals from an organic farm. In addition, sera were obtained from an additional 58 healthy animals to act as extra controls: 30 serum samples from animals aged less than 30 months (8 Friesians and 21 Hereford-Friesian and 1 Charolais-Friesian crossbreeds, the crossbreeds being raised for meat production) and 28 serum samples from animals aged more than 30 months, all of which were dairy Friesians. The animals were raised on a farm where no case of BSE had been reported and were kept under organic farming conditions, with winter feeds consisting of hay and grains but no MBM supplements. Serum samples were obtained during annual herd testing for brucellosis.

Bacterial cultures. *A. calcoaceticus* (NCIMB 10694) and *A. tumefaciens* (NCIMB 9036) were obtained from National Collections of Industrial and Murine Bacteria, Ltd. (Aberdeen, Scotland), and *E. coli* (NCTC 9002) was provided by the Department of Microbiology at King's College. IgA and total Ig (IgG + IgA + IgM) antibodies were measured by ELISA. Cultures were grown in 2-liter flasks on an orbital shaker for 16 h at 37°C for *E. coli* and for 2 days at 30°C for *A. calcoaceticus* and *A. tumefaciens* in 200 ml of nutrient broth (Oxoid; 25 g/liter). Flasks were inoculated with 10 ml of the corresponding starter culture and were left shaking at 37°C for 6 h. Batch culture cells were harvested by centrifugation 6,000 rpm for 20 min at 4°C (Beckman JA-20 rotor, six 250-ml cuvettes). The pellets of cells were then washed three times with 0.15 M phosphate-buffered saline (PBS; pH 7.4) before being finally resuspended in 20 ml of PBS. A stock solution of the suspension was prepared by diluting in 0.05 M carbonate buffer (pH 9.6) to give an optical density (OD) reading of 0.25 (10^6 bacterial cells/ml) on the spectrophotometer (Corning Model 258).

ELISA. ELISAs were carried out as previously described (20). Briefly, ELISA plates were coated (5 μg/well) with neurofilaments prepared from bovine spinal cord (Sigma), myelin basic protein obtained from bovine brain (Sigma), or bacterial suspension (200 μl/well) overnight at 4°C, and the nonspecific sites were blocked with PBS containing 0.1% Tween and 0.2% ovalbumin (Grade III; Sigma), plates were washed, and a 1/200 dilution of test or control serum was added. The plates were incubated at 37°C for 2 h, were washed, and rabbit antibovine alpha-chain-specific horseradish peroxidase conjugate (1/3,000)(Bethyl Laboratories, Ltd.) or rabbit anticow Ig (IgG + IgA + IgM)-horseradish peroxidase

conjugate (1/4,000)(Dako Ltd.) was added. The plates were reincubated for 2 h, were washed, and a substrate solution of 0.5 mg of 2,2'-azinobis(3-ethylbenzthiazoline-6-sulphonic acid)(ABTS; Sigma) per ml in citrate-phosphate buffer (pH 4.1) containing 0.98 mM H_2O_2 (Sigma) was added to each well. The reaction was stopped with a 2-mg/ml solution of sodium fluoride (Sigma), the plates were read at 630 nm on a microtiter plate reader (Dynatech MR 600), and results were expressed as OD units +/– ± standard errors (SE). Each serum sample was tested in duplicate. All studies were blind, in that the tester did not know which were test or control sera. The mean OD units of IgA or total Ig antibodies in serum samples from BSE-positive animals resulting from tests against the two autoantigens and three different microorganisms were compared to the corresponding control groups by using Student's *t* test.

Furthermore, triplicate ELISA studies were carried out in serial doubling dilutions of three selected BSE serum samples which had high, medium, and low reactivities to the respective antigens bovine neurofilaments, bovine myelin, and *A. calcoaceticus*.

Absorption studies. Serum samples from six animals with BSE and high antibody levels to *A. calcoaceticus*, bovine myelin, and neurofilaments were selected for absorption studies. A suspension of *A. calcoaceticus*, OD 1.60 at 540 nm, was sonicated using an MSE Soni prep 150 with a 1/2-in probe, amplitude 10 to 14, for five 1-min bursts. Serum samples (200 μl) were absorbed with sonicated bacteria (25 μl) in a plastic tube and were rotated gently overnight at 4°C. The absorption was repeated until the antibacterial antibody levels for each sample were below the mean value for healthy controls when measured by ELISA (mean OD ± SE). Absorbed sera were then retested for reactivity against bovine myelin and neurofilaments, as previously described.

RESULTS

▲ Top
▲ Abstract
▲ Introduction
▲ Materials and Methods
• Results
▼ Discussion
▼ References

Measurement of autoantibodies to brain components. Elevated levels of IgA autoantibodies to bovine neurofilaments (Fig. 1a) and bovine myelin (Fig. 1b) were found in the 29 animals with BSE (respective mean ODs ± SEs, 0.451 ± 0.029 and 0.260 ± 0.019) when compared to 18 animals free of BSE (0.149 ± 0.009; $P < 0.001$) (0.100 ± 0.0012; $P < 0.001$), 30 organically raised cows less than 30 months of age (0.149 ± 0.007; $P < 0.001$) (0.078 ± 0.005; $P < 0.001$), and 28 organically raised cows greater than 30 months of age (0.157 ± 0.006; $P < 0.001$) (0.078 ± 0.005; $P < 0.001$).

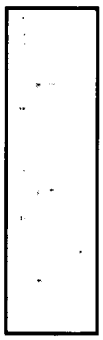

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FIG. 1. IgA antibody titers (bar = mean) for 30 control serum samples from cows aged less than 30 months (A<30m), 28 control serum samples from cows aged more than 30 months (A>30m), and 18 control serum samples from cows not having BSE at postmortem compared to 29 serum samples from cows with BSE at postmortem when tested against bovine neurofilaments (a), bovine myelin (b), and *A. calcoaceticus* (c). Dashed line represents 95% confidence limits for mean of control as given by A<30M + A>30M – results of the one-tailed test.

Elevated levels of IgA antibodies to whole *A. calcoaceticus* bacteria (Fig. 1c) were found in the 29 BSE-affected cattle (0.737 ± 0.022) when compared to 18 animals free of BSE (0.416 ± 0.024; $P < 0.001$), 30 organically raised cows less than 30 months of age (0.409 ± 0.009; $P < 0.001$), and 28 organically raised cows greater than 30 months of age (0.432 ± 0.029; $P < 0.001$). Absorption of BSE sera with sonicated *A. calcoaceticus* reduced autoantibodies to bovine myelin and neurofilaments almost to the levels found in control sera (Table 2), although some activity to neurofilaments remained.

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TABLE 2. Levels of IgA before and after ELISA absorption with bacteria (mean OD ± SE)

Measurement of antibacterial antibodies. Antibodies to *A. calcoaceticus* of total Ig (IgG + IgA + IgM) were significantly elevated in the sera from animals with BSE (0.99 ± 0.05) (Fig. 2a) compared to CVL controls (0.65 ± 0.06; $P < 0.001$) and organic farming controls, either in animals greater than 30 months of age (0.57 ± 0.03; $P < 0.001$) or in animals less than 30 months of age (0.53 ± 0.02; $P < 0.001$). There was no significant difference between the CVL controls and the organic farming controls aged more than 30 months, but there was a small, statistically significant difference when compared with the sera from animals aged less than 30 months ($P < 0.05$). However, there was no significant difference in the level of anti-*A. calcoaceticus* antibodies between organic farming animals aged more than 30 months when these animals were compared to those aged less than 30 months. There was no significant difference between the BSE sera and the three control groups in the levels of either anti-*A. tumefaciens* (Fig. 2b) or anti-*E. coli* antibodies (Fig. 2c).

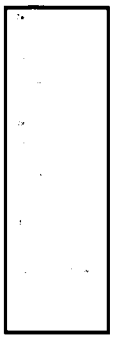

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FIG. 2. Total antibody titers (bar = mean) for 30 control serum samples from cows aged less than 30 months (A<30m), 28 control serum samples from cows aged more than 30 months (A>30m), and 18 control serum samples from cows not having BSE at postmortem compared to 29 serum samples from cows with BSE at postmortem. Total antibody titers were measured against *A. calcoaceticus* (a), *A. tumefaciens* (b), and *E. coli* (c). Dashed line represents 95% confidence limits for mean of controls by the same formula as in the legend to Fig. 1.

Measurement of serial dilutions. ELISA estimations of three BSE serum samples which had high, medium, and low respective reactivities to the following antigens are shown: bovine neurofilaments (Fig. 3a), bovine myelin (Fig. 3b), and *A. calcoaceticus* (Fig. 3c).

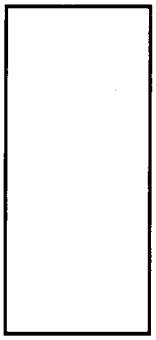

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FIG. 3. Serial doubling dilutions (mean ± SE) of high-, medium-, and low-reactivity BSE sera against bovine neurofilaments (a), bovine myelin (b), and *A. calcoaceticus* (c).

In each case, the high-titer serum reacted with a dilution of up to 1/6,400 of its respective antigen, whereas the medium- and low-titer sera gave lower readings.

DISCUSSION

▲ Top
▲ Abstract
▲ Introduction
▲ Materials and Methods
▲ Results
• Discussion
▼ References

Elevated levels of autoantibodies to bovine neurofilaments and myelin, as well as elevated levels of specific antibodies to *A. calcoaceticus*, have been shown to be present in BSE-affected cattle when compared to three different groups of controls, whilst no such elevations have been seen against either *E. coli* or *A. tumefaciens*. This is clearly a specific observation, since the other two species of microorganisms tested did not show such elevations in their antibody levels. The

agent responsible for the production of these specific autoantibodies is unclear, but it would seem that BSE cattle have been exposed to *A. calcoaceticus*. Whether this implies a link to the neurological features of the disease remains to be determined. This interesting observation requires confirmation with a larger sample of sera from animals with BSE selected from different parts of the United Kingdom and with the analysis carried out with different species of *Acinetobacter*. Furthermore, such sera should be tested against other bacteria commonly present in the bowel flora, as well as against peptides derived from the cross-reacting sequences resembling bovine neurofilaments, myelin, and other brain tissues.

A. calcoaceticus is a species of saprophytic and aerobic gram-negative bacteria that is widely distributed in soil and water supplies, but can also be cultured from skin, mucous membranes, and body secretions from both animals and humans. It is relevant to note that *A. tumefaciens* antibodies are not elevated in animals with BSE. This microbe does not have glutamic acid in the cross-reacting epitope when compared to either *Acinetobacter* or bovine myelin (4), and furthermore, it is a plant pathogen of small trees and shrubs, which makes it unlikely that grass-eating animals like cows would have been exposed to it.

One clear result from these studies is that in at least one TSE disease, namely BSE, specific immune responses predominantly involving IgA, suggesting antigenic exposure across a mucosal surface such as the gut, can be demonstrated against a microbe that is found readily in the environment of cattle and which also happens to possess molecular sequences resembling bovine neurofilaments and myelin. Determinations of whether this microbe was introduced into the food chain of cattle following changes in the preparation of winter feeds or has any pathological significance in the development of BSE await further studies.

Autoantibodies to neuronal components have previously been reported in TSEs, especially in patients with kuru and Creutzfeldt-Jakob disease (15) and in animals with natural scrapie (2). The pathological significance of these autoantibodies remains unclear, but there are three human autoimmune diseases in which molecular mimicry occurs between bacterial antigens and self tissues: rheumatic fever (*Streptococcus pyogenes*) (9), rheumatoid arthritis (*Proteus mirabilis*) (18, 21), and ankylosing spondylitis (*Klebsiella*) (3, 6).

Rheumatic fever is the classic model of an autoimmune disease caused by an infection. A bacterial infection of the tonsils by *S. pyogenes* evokes the formation of antibodies which bind to heart tissue, resulting in acute rheumatic fever, because there is molecular mimicry or similarity between cardiac tissues and streptococcal antigens. Furthermore, antistreptococcal antibodies can also bind to the basal ganglia of the brain, thereby evoking abnormal gait movements, and this is known as rheumatic fever chorea or Sydenham's chorea (8). Injection of antistreptococcal antibodies into rabbits will produce abnormal neurological features of disordered gait and postmortem elution from the rabbit basal ganglia will lead to recovery of an antibody with specificity for streptococcal antigens.

A similar neurological disorder could occur in cattle with BSE following the production of anti-*A. calcoaceticus* antibodies, since this microbe possesses antigens resembling brain tissue. Another possibility is that these anti-*A. calcoaceticus* antibodies appeared following damage to brain tissues by

prions, a situation that frequently occurs in patients with burns who develop antiskin antibodies or following a myocardial infarction, when anticardiac autoantibodies can be detected. A third possibility is that direct infection of brain tissues could occur, similar to the recent observation that *Chlamydia* microbes can be isolated from the cerebrospinal fluid of patients with multiple sclerosis (16). Further studies are required to determine whether anti-*A. calcoaceticus* antibodies exhibit cytotoxic responses against neurons, involving complement activation and NK cells, and to assess the possible relationships between normal (PrP^{c}) and abnormal (PrP^{sc}) prions, *A. calcoaceticus*, and brain autoantibodies in BSE. The mechanism responsible for these serological observations remains unclear, but at least these results confirm and extend the observations of Gajdusek's group that autoantibodies to brain components are present in TSEs.

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FOOTNOTES

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REFERENCES

▲ Top
▲ Abstract
▲ Introduction
▲ Materials and Methods
▲ Results
▲ Discussion
• References

1. **Anderson, R. M., C. A. Donnelly, N. M. Ferguson, M. E. S. Woolhouse, C. S. Watt, H. J. Udy, S. Mawhinney, S. P. Dunstan, and T. R. E. Southwood.** 1996. Transmission dynamics and epidemiology of BSE in British cattle. Nature **382:**779-788[Medline].
2. **Aoki, T., C. J. Gibbs, J. Sotello, and D. C. Gajdusek.** 1982. Heterogenic autoantibody against neurofilament protein in sera of animals with experimental Kuru, Creutzfeldt-Jakob disease and natural scrapie infection. Infect. Immun. **38:**316-324[Medline].
3. **Ebringer, A.** 1992. Ankylosing spondylitis is caused by Klebsiella: evidence from immunogenetic, microbiologic and serologic studies. Rheum. Dis. Clin. N. Am. **18:**105-121[Medline].
4. **Ebringer, A., S. J. Pirt, C. Wilson, P. Cunningham, C. Thorpe, and C. Ettelaie.** 1997. Bovine

spongiform encephalopathy: is it an autoimmune disease due to bacteria showing molecular mimicry with brain antigens? Environ. Health Perspect. **105:**1172-1174[Medline].

5. **Eylar, E. H., J. Caccam, J. J. Jackson, F. C. Westfall, and A. B. Robinson.** 1970. Experimental allergic encephalomyelitis: synthesis of disease-inducing site of the basic protein. Science **168:**1220-1223[Medline].
6. **Fielder, M., S. J. Pirt, I. Tarpey, C. Wilson, P. Cunningham, C. Ettelaie, A. Binder, S. Bansal, and A. Ebringer.** 1995. Molecular mimicry and ankylosing spondylitis: possible role of a novel sequence in pullulanase of *Klebsiella pneumoniae*. FEBS Lett. **369:**243-248[Medline].
7. **Forloni, G., N. Angeretti, R. Chiesa, E. Monzani, M. Salmona, O. Bugiani, and F. Tagliavini.** 1993. Neurotoxicity of a prion protein fragment. Nature **362:**543-546[Medline].
8. **Husby, G., I. Van de Rijn, J. B. Zabriskie, Z. H. Abdin, and R. C. Williams.** 1976. Antibodies reacting with cytoplasm of subthalamic and caudate nuclei neurons in chorea and acute rheumatic fever. J. Exp. Med. **144:**1094-1110[Abstract].
9. **Kaplan, M. H., and M. Meyeserian.** 1962. An immunological cross-reaction between group A *streptococcal* cells and human heart tissue. Lancet **i:**706-710.
10. **Prineas, J., C. S. Raine, and H. Wisniewski.** 1969. An ultrastructural study of experimental demyelination. III. Chronic experimental allergic encephalomyelitis in the nervous system. Lab. Investig. **21:**472-483[Medline].
11. **Prusiner, S. B.** 1982. Novel proteinaceous infectious particles cause scrapie. Science **216:**136-143[Medline].
12. **Prusiner, S. B.** 1994. Biology and genetics of prion diseases. Ann. Rev. Microbiol. **48:**655-686[Abstract].
13. **Purdey, M.** 1994. Are organophosphate pesticides involved in the causation of bovine spongiform encephalopathy (BSE)? Hypothesis based upon literature review and limited trials on BSE cattle. J. Nutr. Med. **4:**43-82.
14. **Raine, C. S., D. H. Snyder, M. P. Valsamis, and S. H. Stone.** 1974. Chronic experimental allergic encephalomyelitis in inbred guinea pigs. An ultrastructural study. Lab. Investig. **31:**369-380[Medline].
15. **Sotello, J., C. J. Gibbs, and D. C. Gajdusek.** 1980. Autoantibodies against axonal neurofilaments in patients with Kuru and Creutzfeldt-Jakob disease. Science **210:**190-193[Medline].
16. **Sriram, S., C. W. Stratton, S. Yao, A. Tharp, L. Ding, J. D. Bannan, and W. M. Mitchell.** 1999. *Chlamydia pneumoniae* infection of the central nervous system in multiple sclerosis. Ann. Neurol. **46:**6-14[Medline].
17. **Thomas, F. P., W. Trojaborg, C. Nagy, M. Santoro, S. A. Sadiq, N. Latou, and A. P. Hays.** 1991. Experimental autoimmune neuropathy with anti-GM1 antibodies and immunoglobulin deposits at the nodes of Ranvier. Acta Neuropathol. **82:**378-383[Medline].
18. **Tiwana, H., C. Wilson, A. Alvarez, R. Abuknesha, S. Bansal, and A. Ebringer.** 1999. Cross-reactivity between the rheumatoid arthritis-associated motif EQKRAA and structurally related sequences found in *Proteus mirabilis*. Infect. Immun. **67:**2769-2775[Abstract/Full Text].
19. **Towner, K. J.** 1997. Clinical importance and antibiotic resistance of *Acinetobacter* species. J. Med. Microbiol. **46:**721-746[Medline].
20. **Will, R. G., J. W. Ironside, M. Zeidler, S. N. Cousens, K. Estibeiro, K. Alperovitch, S. Poser, M. Pocchiari, A. Hofman, and P. G. Smith.** 1996. A new variant of Creutzfeldt-Jakob disease in the U.K. Lancet **347:**921-925[Medline].
21. **Wilson, C., A. Ebringer, K. Ahmadi, J. Wrigglesworth, H. Tiwana, M. Fielder, A. Binder, C. Ettelaie, P. Cunningham, C. Joannou, and S. Bansal.** 1995. Shared amino acid sequences

between major histocompatibility complex class II glycoproteins, type XI collagen and *Proteus mirabilis* in rheumatoid arthritis. Ann. Rheum. Dis. **54:**214-220.

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Biology and Genetics of Prion Diseases

Stanley B. Prusiner

Enriching fractions from Syrian hamster (SHa) brain for scrapie prion infectivity led to the discovery of the prion protein (PrP). Prion diseases include scrapie of sheep, bovine spongiform encephalopathy (BSE) of cattle, as well as Creutzfeldt-Jakob disease (CJD), Gerstmann-Strä ussler-Scheinker disease (GSS), and fatal familial insomnia (FFI) of humans. Discovery of mutations in the PrP genes of humans with familial CJD, GSS, and FFI established that prion diseases are both genetic and infectious. Many lines of evidence have converged to argue that infectious prions are composed largely, if not entirely, of PrPsc molecules. Mice overexpressing mutant and wild-type transgenes develop neurologic illnesses spontaneously and produce prions as demonstrated by serial transmission of disease in rodents after inoculation of brain extracts. Although these and many other findings argue that prions are devoid of nucleic acid, the molecular basis of prion strains remains enigmatic. The formation of PrPsc from PrPc is a posttranslational process involving the conversion of {alpha} -helices into {beta} -sheets. This conformational change in PrP appears to be the fundamental event that underlies prion propagation and the pathogenesis of prion diseases. The unique features of prion structure and propagation differentiate prions from all other transmissible pathogens.

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- Kochneva-Pervukhova, N. V., Paushkin, S. V., Kushnirov, V. V., Cox, B. S., Tuite, M. F., Ter-Avanesyan, M. D. (1998). Mechanism of inhibition of Psi + prion determinant propagation by a mutation of the N-terminus of the yeast Sup35 protein. *EMBO J.* 17: 5805-5810 [Abstract] [Full Text]
- Tiwana, H., Wilson, C., Pirt, J., Cartmell, W., Ebringer, A. (1999). Autoantibodies to Brain Components and Antibodies to Acinetobacter calcoaceticus Are Present in Bovine Spongiform Encephalopathy. *Infect. Immun.* 67: 6591-6595 [Abstract] [Full Text]
- Vey, M., Pilkuhn, S., Wille, H., Nixon, R., DeArmond, S. J., Smart, E. J., Anderson, R. G. W., Taraboulos, A., Prusiner, S. B. (1996). Subcellular colocalization of the cellular and scrapie prion proteins in caveolae-like membranous domains. *Proc. Natl. Acad. Sci. U. S. A.* 93: 14945-14949 [Abstract] [Full Text]
- Kaneko, K., Vey, M., Scott, M., Pilkuhn, S., Cohen, F. E., Prusiner, S. B. (1997). COOH-terminal sequence of the cellular prion protein directs subcellular trafficking and controls conversion into the scrapie isoform. *Proc. Natl. Acad. Sci. U. S. A.* 94: 2333-2338 [Abstract] [Full Text]
- Derkatch, I. L., Bradley, M. E., Liebman, S. W. (1998). Overexpression of the SUP45 gene encoding a Sup35p-binding protein inhibits the induction of the de novo appearance of the [PSI+]

prevented from occurring elsewhere. To do that, knowledge of BSE and other members of the group should be widely dispersed and it is the purpose of this issue to do just that.

Publication Types:
- Review
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1: *Rev Sci Tech* 1992 Jun;11(2):605-34 Related Articles, Books, LinkOut

Sub-acute, transmissible spongiform encephalopathies: current concepts and future needs.

Bradley R, Matthews D

Ministry of Agriculture, Fisheries and Food, Central Veterinary Laboratory, Addlestone, Surrey, United Kingdom.

The first diagnosis of bovine spongiform encephalopathy (BSE) in the United Kingdom in 1986 was to stimulate the most intensive epidemiological study of any animal disease of all time in that country. It led also to the initiation of a broad-based research programme with an international flavour. This principally involved scientists and veterinarians in Europe (especially the United Kingdom) and the United States of America, especially those with experience of slow infections in general and experimental scrapie in particular. This final chapter highlights some of the significant discoveries made in the study of BSE and related diseases of this group but also emphasises the deficits in knowledge which need to be corrected before such diseases as scrapie in sheep and goats can be brought under control. The benefits resultant upon effective disease control will be manifest as improvement in animal production, welfare and, importantly, the removal of trading barriers currently in place to protect countries in which diseases such as BSE and scrapie do not exist. Of key importance is the development of a simple, cheap and effective diagnostic test for use in the live animal before the onset of clinical signs. This will be difficult since the nature of the causal agents is uncertain and none provokes either a detectable immune response or inflammatory reaction in the host. The earlier chapters, written by acknowledged specialists from around the world, deal with the specific diseases in detail and all present some of the most recent knowledge available. Here the authors emphasise the important role that major national and international agencies have in effecting the highest level of control possible in the absence of key information. International collaboration with countries in which these diseases exist, and as well as those where they are absent, is of paramount importance. It is essential that the BSE epidemic which has severely affected the cattle industry of the United Kingdom is not allowed to happen in developing countries. Whereas the former has implemented stringent control measures based on scientific knowledge and is well on the way to eradicating the disease, the latter could have much greater difficulty in establishing control. The answer is clear. BSE must be

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Novel proteinaceous infectious particles cause scrapie.

Prusiner SB

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Novel Proteinaceous Infectious Particles Cause Scrapie

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Summary. After infection and a prolonged incubation period, the scrapie agent causes a degenerative disease of the central nervous system in sheep and goats. Six lines of evidence including sensitivity to proteases demonstrate that this agent contains a protein that is required for infectivity. Although the scrapie agent is irreversibly inactivated by alkali, five procedures with more specificity for modifying nucleic acids failed to cause inactivation. The agent shows heterogeneity with respect to size, apparently a result of its hydrophobicity; the smallest form may have a molecular weight of 50,000 or less. Because the novel properties of the scrapie agent distinguish it from viruses, plasmids, and viroids, a new term "prion" is proposed to denote a small proteinaceous infectious particle which is resistant to inactivation by most procedures that modify nucleic acids. Knowledge of the scrapie agent structure may have significance for understanding the causes of several degenerative diseases.

A major, unanswered question in molecular biology concerns the chemical structure of the scrapie agent. Until recently, mysteries surrounding the scrapie agent were so commonplace that investigators had come to accept rather than question its enigmatic properties. The scrapie agent causes a degenerative disorder of the central nervous system (CNS) in sheep and goats (*1*).

The extraordinary resistance of the scrapie agent to Formalin was responsible for the inadvertent inoculation of sheep in Scotland. Eighteen thousand animals were vaccinated against louping ill virus with a formalin-treated suspension of ovine brain and spleen that, as was shown subsequently, had been contaminated with the scrapie agent (*2*). Two years later, 1500 sheep developed scrapie. Subsequently, studies on CNS diseases (including scrapie) of sheep provided the foundation for Sigurdsson's concept of slow infections (*3*). In 1959, Hadlow suggested that kuru, a CNS degenerative disease of New Guinea highlanders, might be similar to scrapie because the pathologies of these disorders share many features (*4*). The transmission of kuru to chimpanzees in 1965 by Gajdusek, Gibbs, and Alpers forced a major reconsideration of the etiology of all degenerative disorders and made scrapie a subject of intense medical interest (*5*). Subsequently, Creutzfeldt-Jakob disease (CJD), a progressive, presenile dementia, was shown by Gibbs, Gajdusek, and co-workers to be caused by a transmissible agent (*6*, *7*).

A recent study suggests that there may be similarities between the agents causing scrapie and CJD (*8*). Goats inoculated with brain tissue from demented patients dying of CJD developed a neurological disorder 3 to 4 years after inoculation (Fig. 1). Five out of ten CJD inocula have produced disease in goats (*9*). Experimental CJD in goats is indistinguishable both clinically and neuropathologically from natural scrapie. Monkeys have been used as a common experimental host for scrapie and CJD; curiously, chimpanzees are susceptible to CJD but not scrapie (*10*). Numerous attempts to link scrapie epidemiologically to CJD have been unsuccessful (*11*). At present, there is no direct evidence that the scrapie agent causes disease in humans.

In contrast to CJD which occurs worldwide, kuru is found only in a small mountainous region of Papua New Guinea. Epidemiological studies of kuru provide evidence for incubation periods of 20 to 30 years (*12*, *13*). Although considerable evidence implicates cannibalism in the spread of kuru, no direct observations of cannibalistic acts in the "endemic" region have been recorded. Attempts to transmit kuru by feeding infected brain tissue to chimpanzees have been unsuccessful although one monkey developed a kuru-like illness 36 months after oral ingestion of the kuru agent (*14*). In contrast, goats fed scrapie-infected tissue frequently develop disease (*15*). Recently, we have taken advantage of the natural cannibalistic activities f hamsters to develop an experimental model of scrapie transmitted by cannibalism (*16*). Oral transmission of the scrapie agent appears to be extremely inefficient. Cannibalism requires a dose of agent 10^9 times greater than that needed to produce scrapie by intracerebral injection. These results provide compelling evidence for oral transmission of th scrapie agent and may offer new insights into the spread of kuru by cannibalism among the Fore people and their neighboring tribes.

Bioassay of the Scrapie Agent

Studies on the scrapie, kuru, and CJD agents have been greatly limited by the slow, tedious, and costly bioassays used to detect these agents. Since tissue culture systems are not available for the replication and assay of these agents and they appear to be nonantigenic in their native forms, animal bioassays must be used. For many years all assays for the scrapie agent were performed in sheep and goats (*17*). In 1961, transmission of the scrapie agent to mice ransformed research (*18*), but the murine end-point titration assay was still heroic. Quantifying a single sample required eight to ten serial tenfold dilutions and injection of each dilution into six mice (*19*). Then 50 to 60 mice were held for 1 year and examined weekly for signs of scrapie. The number of animals developing scrapie at the highest dilution was used to calculate an end point. The time required for titration of a sample was reduced to 200 days when a more rapid form of the disease in hamsters was discovered (*20*, *21*).

Several investigators have estimated scrapie titers by measuring the time interval from inoculation to onset of illness (incubation period) in mice (*22*, *23*). Reluctance to refine such measurements has prevented its wide use in mice.

With hamsters, studies on the scrapie agent have been accelerated by development of a bioassay based on measurements of incubation time (*24*, *25*). It is now possible to assay samples with the use of four animals in 60 to 70 days if the titers of the scrapie agent are high. As is shown in Fig. 2, the interval from inocu-

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lation t onset of illness (y) was inversely proportional to the dose injected intracerebrally into random bred weanling Syrian hamsters. The logarithm of the mean interval ($\bar{y}$) in days minus a time factor of 40 is a linear function of the logarithm of th dose over a wide range; the time factor was determined by maximizing the linear relation between the time interval and dose. With a factor of 40, the regression coefficient of the line is 0.87. A similar analysis was performed for the time interval from inoculation to death (z). With a time factor of 61, the regression coefficient of the line is 0.86. Linear relationships were also obtained when the reciprocals of the time intervals were plotted as a function of the logarithm of the dose.

Replication of the Scrapie Agent

The kinetics of scrapie agent replication in hamsters and mice are well documented (*21*, *26*). After intracerebral inoculation of hamsters with 10^7 ID_{50} (median infectious dose) units, about 10^2 ID_{50} units can be recovered in the brain 24 hours later. During the next 50 days the amount of agent in the brain increases to 10^9 ID_{50} units. At this time the agent is widely distributed throughout the brain and no regional differences are apparent (*27*). The neuropathology is minimal and the animals exhibit no neurological dysfunction. During the next 10 to 15 days the animals develop ataxia, difficulty righting themselves from a supine position, generalized tremor, and head bobbing. By 60 to 70 days, vacuolation of neurons and astrogliosis are found throughout the brain, even though the titer of the agent remained constant. Thus, the "pathological hallmarks" of this spongiform encephalopathy do not correlate with the extent of agent replication. In addition, the spongiform pathology that characterizes kuru and CJD is inconspicuous in natural scrapie (*28*).

Hypothetical Structures for the Scrapie Agent

Investigators have been aware of the unusual properties of the scrapie agent for more than three decades. Hypotheses on the chemical structure of the scrapie agent have included: sarcosporidia parasite (*29*), "filterable" virus (*30*), small DNA virus (*31*), replicating protein (*32*), replicating abnormal polysaccharide within membranes (*33*), DNA subvirus controlled by a transmissible linkage substance (*34*), provirus consisting

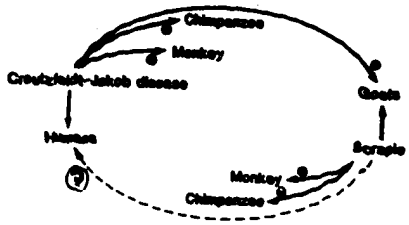

Fig. 1. Experimental relation between Creutzfeldt-Jakob disease of humans and scrapie of goats and sheep.

of recessive genes generating RNA particles (*35*), naked nucleic acid similar to plant viroids (*36*), unconventional virus (*12*, *37*), aggregated conventional virus with unusual properties (*38*), replicating polysaccharide (*39*), nucleoprotein complex (*40*), nucleic acid surrounded by a polysaccharide coat (*41*), spiroplasma-like organism (*42*), multicomponent system with one component quite small (*43*), and membrane-bound DNA (*43*, *44*).

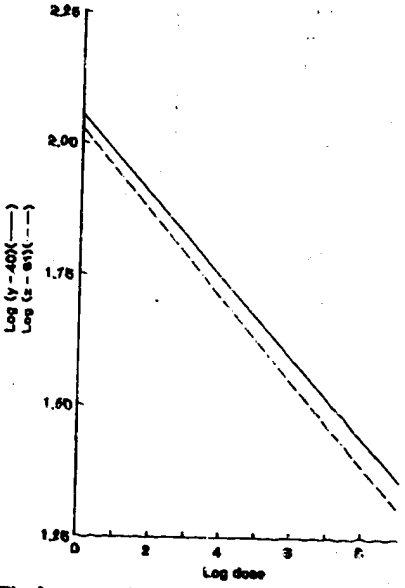

Fig. 2. Assay of the scrapie agent by measurements of the incubation time interval. The intervals from inoculation to onset of illness (y) and to death (z) are plotted as a function of the injected dose. Equations were written to describe these linear functions which relate the titer of the inoculum to the time intervals both from inoculation to onset of illness (Eq. 1) and from inoculation to death (Eq. 2):

$$\text{Log } T_y = 26.66 - (12.99) \log (\bar{y} - 40) - \log D \quad (1)$$

$$\text{Log } T_z = 25.33 - (12.47) \log (\bar{z} - 61) - \log D \quad (2)$$

where T is the titer expressed in ID_{50} units per milliliter, D is the dilution defined as the fractional concentration of the diluted sample, $\bar{y}$ is the mean interval from inoculation to onset of clinical illness in days, and $\bar{z}$ is the mean interval from inoculation to death in days. The most precise estimate of titer is obtained by calculating a weighted average for T_y and T_z.

Purification and Hydrophobicity of the Scrapie Agent

Several investigators have mounted major efforts to purify and characterize the scrapie agent over the past two decades (*45–48*). Early studies suggested that the scrapie agent was distributed throughout virtually all subcellular fractions (*45*, *46*). The interpretation of those observations was complicated by the imprecision of the end-point titrations of the agent. Nevertheless, the scrapie agent was reported to be intimately associated with cellular membranes, and from this association the "membrane hypothesis" evolved (*33*). When various extraction procedures failed to release the agent from membrane fractions, it was concluded that the agent is a replicating membrane fragment that cannot be separated from cellular membranes.

Several different purification procedures have been reported. One involved copurification of the scrapie agent and microsomes (*49*). Another involved isolation of a "membrane-free" fraction after prolonged ultracentrifugation (*50*). This fraction contained 1 to 10 percent of the scrapie agent and was precipitated with ammonium sulfate. Sodium dodecyl sulfate (SDS) gel electrophoresis was used to obtain a further purification (*51*). Although the results of these studies seemed encouraging initially, subsequent work has been disappointing (*52*).

Using equilibrium sucrose and sodium chloride density gradients, Siakotos, Gajdusek, Gibbs, and co-workers have attempted to purify the scrapie agent from murine brain (*53*). They suggested that there was a peak of infectivity at a sucrose density of 1.19 g/cm^3. However, multiple peaks of infectivity were found throughout the gradients, an indication of considerable heterogeneity with respect to density and showing that the technique when applied to crude suspensions of membranous material from brain is probably not useful in isolating the scrapie agent. Other studies from the laboratory of Gajdusek have shown considerable heterogeneity of the agent in metrizamide and cesium chloride density gradients (*54*).

Since the initial purification of many biological macromolecules involves a series of differential centrifugations (*55*), we began our studies on the scrapie agent by defining its sedimentation properties in fixed-angle rotors in order to develop a preparative protocol (*56*). These studies showed that the agent from both murine spleen and brain sedimented over a range of particle sizes from 60*S* to 1000*S* (*19*).

Table 1. Molecular properties of the scrapie agent: evidence that it contains a protein. Abbreviations: PMSF, phenylmethylsulfonylfluoride; DEP, diethyl pyrocarbonate; TX-100, Triton X-100; OGS, octylglucoside; SB 3-14, sulfobetaine 3-14; ET-12H, 1-dodecyl propanediol-3-phosphorylcholine; SDS, sodium dodecyl sulfate; LDS, lithium dodecyl sulfate; Gdn, guanidinium; NP40, Nonidet P-40; Sarkosyl, sodium dodecyl sarcosinate; TCA, trichloroacetic acid; SCN, thiocyanate.

Treatment	Stable	Labile
Protease digestion		Proteinase K, trypsin
Chemical modification		DEP, butanedione, PMSF
Detergents	TX-100, NP40, OGS, SB 3-14, ET-12H, cholate, Sarkosyl	SDS, LDS
Ions	Na^+, K^+, Cl^-, SO_4^{-2}, $EDTA^{-4}$, PO_4^{-3}	Gdn^+, SCN^-, TCA^-
Denaturants		Urea
Organic solvents	Methanol, ethanol	Phenol

On the basis of the information derived from these sedimentation profiles, a partial purification scheme for the murine scrapie agent from spleen was derived (*57*). The preparation was devoid of cellular membranes, and enriched for the scrapie agent 20- to 30-fold with respect to protein and DNA. Studies on the agent by rate-zonal sucrose gradient centrifugation gave sedimentation coefficients for the agent ranging from 40*S* to > 500*S*. Sucrose density gradient centrifugation revealed a particle density ranging from 1.08 to more than 1.30 g/cm^3, an indication that some forms of the agent might be associated with lipids. Further sedimentation studies showed that the agent aggregated with cellular elements on heating the agent in a partially purified fraction (*58*). The agent was stable in nonionic and nondenaturing anionic detergents, but was inactivated by SDS. Free-flow electrophoresis showed that most of the agent has a net negative charge, but significant charge heterogeneity was found.

Heterogeneity of the scrapie agent with respect to size, density, and charge suggested that hydrophobic domains on its surface might be responsible for these phenomena. Such domains are usually formed by the juxtaposition of nonpolar side chains of amino acids within a protein (*57*, *59*).

These initial studies on the murine agent from spleen revealed the complexities of scrapie agent purification. We then developed an improved assay based on measurements of the incubation time. With this new bioassay, we created a purification scheme for the agent from hamster brain, where the titers are highest (*21*). The initial steps of the purification were similar t those for the murine agent (*57*). Deoxycholate extracts (P_4) were digested sequentially with micrococcal nuclease and proteinase K. The digestions were performed at 4°C to prevent aggregation of the agent, which is observed at elevated temperatures (*59*). The digested preparations were then subjected to cholate-Sarkosyl extraction followed by ammonium sulfate precipitation (P_5). Most of the remaining digested proteins and nucleic acids were separated from the scrapie agent by Sarkosyl agarose gel electrophoresis at 4°C (*60*). Such preparations of the eluted scrapie agent (E_6) were 100- to 1000-fold purified with respect to cellular protein (*60*). With these enriched preparations we demonstrated that a protein within the agent is required for infectivity (*60*). Fraction E_6 contains (per milliliter) $10^{6.5}$ to $10^{8.5}$ ID_{50} units of agent, 20 to 50 μg of protein, < 1 μg of DNA, and < 10 μg of RNA.

Since the ratio of particle number to infectivity unit for the scrapie agent is unknown, the extent of purification required to obtain homogeneous preparations is unknown. Each hamster brain contains 10^9 ID_{50} units of the scrapie agent and 100 mg of protein. Assuming an average molecular weight of 50,000 for all proteins and a ratio of particle number to infectivity unit of 1, a 10^7-fold purification will be required to prepare a pure preparation of the agent. However, if the particle to infectivity ratio is 1000, then a 10^4-fold purification will be required. Experience with assaying conventional viruses in animals suggests that the particle to infectivity ratio for the scrapie agent may be considerably greater than 1. These calculations indicate the need for a source of the scrapie agent with considerably higher titers. Attempts to propagate the scrapie agent in cell culture have been disappointing.

The hydrophobicity of the scrapie agent has complicated purification as described above. Further evidence for the hydrophobicity of the scrapie agent comes from its binding to phenyl-Sepharose (*60*). The agent could not be eluted in 8.5*M* ethylene glycol; however, inclusion of 4 percent Nonidet P-40 and 2 percent Sarkosyl in the ethylene glycol eluate resulted in the almost quantitativ recovery of the agent from phenyl-Sepharose. In addition, the hydrophobicity was reflected by diminished titers when detergent was removed from fraction E_6 (*60*). Presumably this decrease in infectivity was due to aggregation.

Not only is the hydrophobic nature of the scrapie agent important with respect to purification, it may also explain some of its enigmatic properties. That hydrophobic interactions increase with elevated temperature may be reflected in the extreme heat stability of the agent (*47*). Numerous unsuccessful attempts to detect antibodies against the native scrapie agent in fraction E_6 might be explained by its hydrophobicity (*61*). Hydrophobic proteins in their native state are sometimes poor antigens (*62*). An alternative (and attractive) explanation for the apparent lack of immunogenicity of the scrapie agent evolves from the possibility that the agent may be closely related to a normal cellular protein to which the host does not produce antibodies.

Scrapie Agent Contains Protein

Six separate and distinct lines of evidence show that the scrapie agent contains a protein that is required for infectivity: (i) inactivation as a result of digestion with proteinase K, (ii) inactivation by chemical modification with diethyl pyrocarbonate, (iii) inactivation by SDS, (iv) inactivation by chaotropic salts such as guanidinium thiocyanate, (v) inactivation by phenol, and (vi) inactivation by urea (*60*). The cumulative evidence for a protein within the scrapie agent appears to be compelling (Table 1).

Digestion with crystalline proteinase K inactivated the scrapie agent from hamster brain (*60*); the decrease in titer was a function of enzyme concentration, temperature, and time of digestion. Prior treatment of proteinase K with the protease inhibitor phenylmethylsulfonylfluoride (PMSF) completely abolished the protease-catalyzed degradation of the agent. Digestion with trypsin also destroyed the scrapie agent. The protease sensitivity of the scrapie agent was revealed only after considerable purification (fraction E_6). Other investigators have occasionally observed decreases in scrapie titers after addition of proteases (*48*, *63*).

Carbethoxylation by diethyl pyrocarbonate also inactivated the purified scrapie agent (*60*, *64*), but activity was restored by treatment with hydroxylamine. This reversibility of the inactive, chemically modified agent provides a further

Table 2. Resistance of the scrapie agent to procedures that attack nucleic acids. Abbreviations: AMT, 4'-aminomethyl-4,5',8-trimethylpsoralen; HEP, 1-α-4' hydroxyethylpsoralen; HMT, 4'hydroxymethyl 4,5',8-trimethylpsoralen; MMT, 4'-methoxymethyl-4,5',8-trimethylpsoralen; TMP, 4,5',8-trimethylpsoralen.

Procedure	Resistant	Labile	Possible explanations
*p*H	H^+	OH^-	Hydrolysis of RNA genome, denaturation of dsDNA genome, or protein denaturation
Nucleases	Ribonucleases, deoxyribonucleases		Enzymes unable to penetrate protein shell
UV irradiation	254 nm		Shielded by protein shell or no critical nucleotide dimers formed
Divalent cation hydrolysis	Zn^{2+}		Ions unable to penetrate protein shell
Psoralen photoreaction	AMT, HEP, HMT, MMT, TMP		Monoadducts of single-stranded genome do not inactivate or psoralens unable to penetrate protein shell
Chemical modification	Hydroxylamine		Nucleophiles react only with surface protein and are unable to penetrate the shell or react minimally with double-stranded genome

argument for a protein target. More recent data on chemical modification indicate that the scrapie agent is also inactivated by 10 m*M* butanedione and 2 m*M* PMSF. Butanedione modifies arginine (*65*), lysine, and histidine residues; modifications of one or more of these amino acid residues may explain its effect on the scrapie agent.

Three reagents used to denature proteins and isolate biologically active nucleic acids (*66*) also inactivate the agent. First, SDS diminished the scrapie agent titer when the ratio of SDS (grams) to protein (grams) exceeded 1.8 (*24*). In contrast, the agent was stable in various nondenaturing ionic and nonionic detergents (Table 1). Simultaneous addition of a nonionic detergent and SDS to a preparation containing the scrapie agent prevented the inactivation observed with SDS alone. Second, studies with chaotropic ions have shown that in low concentrations they inactivate the agent (*24*, *67*). Irreversible inactivation of the agent was found upon exposure to 1*M* guanidinium thiocyanate at 4°C for 3 hours. Higher concentrations of less potent chaotropic salts were required to achieve irreversible inactivation. Third, phenol, useful in the isolation of nucleic acids, inactivates the scrapie agent (*68*). In contrast, the agent is stable in methanol and ethanol, but is readily precipitated. Extraction with phenol, a potent denaturant of protein, under various salt and *p*H conditions destroyed infectivity (*24*). In the above studies partially purified preparations were first digested with proteinase K to prevent the formation of an interface in which the agent might be trapped. We have attempted t restore scrapie agent infectivity from phenol extracted preparations by incorporation int liposomes and by transfection int cultured cells. Using reverse-phase liposome formation (*69*), no infectivity was recovered from the aqueous phase, dialyzed phenol phase, or combination of these two phases (*70*). Preparations of phenol extracted DNA and RNA from scrapie-infected murine spleen failed to produce infectious scrapie agent upon transfection of L cells (*71*). Similar transfection experiments with murine fetal brain cells and embryonic fibroblasts also failed to produce infectious agent (*72*). From all of these studies with chemical reagents that denature proteins but permit isolation of biologically active nucleic acids, we conclude that denaturation of a protein within the scrapie agent leads to inactivation of the infectious particle. Moreover, CJD agents adapted to guinea pigs and mice are also inactivated when extracted with phenol (*73*).

Hunter and co-workers showed that exposure of the scrapie agent to 6.0*M* urea decreased the titer by a factor of 100 (*74*). This high concentration of urea could have denatured protein or nucleic acid. We have found that exposure of the scrapie agent in partially purified fractions to 3*M* urea at 4°C decreases the titer by a factor of 50 (*70*). Removal of the urea after 2 hours was not accompanied by a return of infectivity. This observation contrasts with other findings where removal of the KSCN was accompanied by an apparent return of infectivity (*67*). Whether urea or cyanate ions are responsible for the loss of scrapie infectivity in these experiments (*75*) is not known. From our data the most likely target within the scrapie agent for denaturation by urea is a protein.

The functions of a protein or proteins within the scrapie agent are unknown. The hydrophobicity f the protein should allow it t penetrate membranes, but whether or not there are specific receptors on cell surfaces to which the scrapie protein might bind is unkn wn. Studies on transmission of scrapie by cannibalism in hamsters suggest that the scrapie agent is transported across epithelial cells and then presumably enters the bloodstream (*16*). Manuelidis and coworkers have found the CJD agent in white blood cells (*76*). Studies by Kimberlin and Walker suggest that the agent may be transported within axons much like rabies virus (*77*). One possibility is that the protein is a polymerase that is necessary for replication of a putative nucleic acid within the agent. This would explain the protein requirement for infectivity and would be similar to negative strand viruses. We also must consider the possibility that the scrapie protein acts as an inducer or as a template for its own synthesis.

Search for Nucleic Acid in Scrapie Agent

In our search for a nucleic acid genome within the scrapie agent, we subjected the agent to changes in *p*H (Table 2). Although other investigators had indicated that the agent was stable over a *p*H range from 2 to 10.5 (*78*), our observations do not agree with some of these earlier studies. We have found that the titer of the scrapie agent is irreversibly reduced by alkali (*67*). The titer was reduced by a factor of 1000 on exposure to *p*H 10 for 1 hour at 4°C or by a factor of 100 on exposure to *p*H 9 for 1 hour at 37°C (*70*). Neutralization with acid did not restore infectivity. In contrast, no loss of infectivity at *p*H 3 was observed over a 16-hour period at 37°C. One interpretation of these studies is that alkali hydrolyzed a few phosphodiester bonds within a scrapie nucleic acid rendering the agent inactive. The covalent backbone in RNA is labile to alkali while that in DNA is generally stable; however, base modifications such as methylation of purines render DNA labile in alkali (*79*). Denaturation of double-stranded DNA (dsDNA) in alkali is also well doc-

umented (*80*). Alternatively, inactivation by alkali under rather mild conditions could be due to protein denaturation. Unfortunately, the lack of specificity in these *p*H stability studies does not allow us to make a definitive statement concerning the presence or absence of a nucleic acid within the scrapie agent.

Over the past 15 years, two techniques with high degrees of specificity have suggested that the scrapie agent might not contain a nucleic acid. The scrapie agent in crude preparations has been found to be resistant to nuclease digestion (*46*, *48*, *59*) and to ultraviolet (UV) irradiation at 254 nm (*81*, *82*). The objection to these studies was that a protective coat prevented nucleases from penetrating the agent, as well as shielding it from radiation.

At several different stages of purification we have searched for susceptibility of the agent to nuclease digestion. No decrease in scrapie infectivity has been observed with micrococcal nuclease, nuclease P_1, deoxyribonucleases I and II, ribonucleases A and T_1, and phosphodiesterases I and II at 10, 100, and 500 μg/ml f r 3 to 30 hours at 37°C. Ribonucleases III and H at 1 and 10 unit/ml also showed no effect. Although nuclease sensitivity has been described for the scrapie agent (*44*), we have been unable to confirm this observation (*52*).

The complete lack of scrapie agent sensitivity to nucleases in view of inactivation by proteases is of interest. Numerous viruses are resistant to nucleases; presumably, these enzymes do n t penetrate the viral protein coats (*83*). In c ntrast, addition of ribonuclease A at 0.1 μg/ml to a crude nucleic acid extract containing potato spindle tuber viroid (PSTV) decreased the PSTV titer by a factor of $>10^6$ in 1 hour at 25°C (*84*). Hydrolysis of a single phosphodiester bond within a viroid probably inactivates it (*85*, *86*). There are many examples of proteins that retain their biological activities after limited proteolysis (*87*). We do not know in the case of the scrapie agent how many peptide bonds must be cleaved to cause inactivation.

Studies with the optically clear fraction E_6 have confirmed the resistance of the scrapie agent to UV-inactivation (*81*, *82*). Fractions S_2, P_5, and E_6 were irradiated at 254 nm with increasing doses. Although no inactivation of the agent in fraction S_2 was observed, a minimal but probably significant decrease was f und in fractions P_5 and E_6 as a function of dose (*88*). The kinetics f inactivation by irradiation at 254 nm suggest a single-hit process. The survival f 37 percent of the scrapie agent in fractions P_5 and E_6 was observed after a UV dose (D_{37}) of 42,000 J/m^2. The resistance of the scrapie agent to irradiation at 254 nm is compared to that observed for viruses and viroids in Table 3. Clearly, the inactivation of the scrapie agent at these extreme energy levels indicates a photochemistry of a far different nature from that observed for virus inactivation through the formation of thymine or uracil dimers. Proteins are relatively resistant to irradiation at 254 nm (*89*) and are probably the target within the scrapie agent in these irradiation studies.

Table 3. Inactivation f small infectious agents by UV irradiation at 254 nm.

Example	D_{37} (J/m^2)*
Bacteriophage T2	4
Bacteriophage S13	20
Bacteriophage φX174	20
Rous sarcoma virus	150
Polyoma virus	240
Friend leukemia virus	500
Murine leukemia virus	1,400
Potato spindle tuber viroid	5,000
Scrapie agent	42,000

*Data from (*82*, *85*, *88*). D_{37} is the dose of irradiation that permits 37 percent survival.

Observations on the resistance of the scrapie agent to procedures attacking nucleic acids have been extended by means of three other techniques (Table 2). The agent has been incubated at *p*H 7 in the presence of 2 m*M* $Zn(NO_3)_2$ at 65°C f r periods as long as 24 hours without loss of infectivity (*70*). Under these conditions polymers of RNA are completely reduced to mononucleotides, and polymers of DNA undergo considerable hydrolysis (*90*). Photochemical inactivation of the scrapie agent with psoralens was attempted with samples at several levels of purification, both from murine spleen and hamster brain. Five different psoralens of varying degrees of hydrophobicity were used (*91*). It was expected that the most hydrophobic psoralens readily partitioned into the scrapie agent. No inactivation of the scrapie agent was observed with any of these psoralens over a wide range of dosages (*92*). Psoralens may form diadducts upon photoactivation within base-paired regions of nucleic acids and monoadducts within single-stranded regions (*93*). Psoralens have several advantages in searching for a nucleic acid genome: (i) low reactivity with proteins, (ii) penetration of viral protein and lipid coats, and (iii) formation of stable covalent linkages on photoactivation. Psoralens have been found to inactivate numerous viruses, but not, for example, picornaviruses (*94*). Psoralens, like acridine orange and neutral red dyes (*95*), do not penetrate the protein coat of poliovirus. Photoadducts with viral RNA were formed when psoralens or the above tricyclic dyes were added to cultured cells replicating the poliovirus.

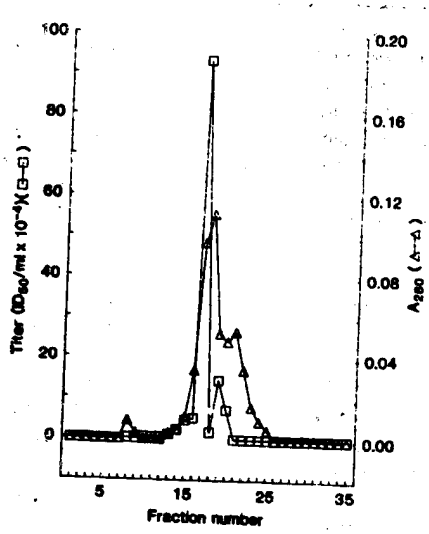

Fig. 3. Molecular sieve chromatography of the scrapie agent in sulfobetaine 3-14. Fraction P_5 was exposed to 10 percent (weight to volume) sulfobetaine 3-14 for 16 hours at 4°C and then chromatographed in 60 m*M* tris-acetate, *p*H 7.2 containing 1 m*M* EDTA and 0.024 percent sulfobetaine 3-14 over a molecular sieve (Toya Soda TSK 4000) column (0.75 cm, inside diameter, by 50 cm, length). The column was developed at 4°C (Varian 5000 HPLC) at 1 ml/min. Fractions (1 ml) were collected and assayed in hamsters by measurements of incubation time intervals. Blue dextran, bovine serum albumin, and tryptophan under identical conditions eluted in fractions 7, 15, and 22, respectively.

In contrast to psoralens, hydroxylamine readily inactivates poliovirus at neutral *p*H (*96*). Hydroxylamine does not generally react with proteins at neutral *p*H, but it does decarbethoxylate modified proteins and it does modify cytosine bases (*97*). At concentrations up to 0.5*M* at neutral *p*H hydroxylamine failed to alter scrapie agent infectivity (*64*). Under these conditions, most animal and plant viruses as well as bacteriophage are inactivated by hydroxylamine (*98*), except for the paramyxoviruses, which are resistant. In contrast, inactivation of the scrapie agent by carbethoxylation upon treatment with diethyl pyrocarbonate was found to be reversible with NH_2OH (*64*).

The extreme resistance f the scrapie agent to inactivation suggests that its structure is different from that of viruses. While there are examples of viruses that are resistant to inactivation by two or even three of the six procedures in Table 2, we are unaware of any viruses which, like the scrapie agent, are resistant by all

of these procedures. However, the possibility must be considered that the putative genome of the scrapie agent is buried within a tightly packed protein shell which excludes nucleases, UV irradiation, Zn^{2+}, psoralens, and NH_2OH. Also, we cannot exclude an unusual nucleic acid with a different base structure or polymer packing that might exhibit the resistant characteristics described for the scrapie agent.

Of interest are studies showing a large oxygen effect upon exposure of the scrapie agent to ionizing radiation (*99*). Viruses and nucleic acids characteristically show a small oxygen effect. Biological membranes and probably lipoproteins show large oxygen effects. The increased sensitivity of the scrapie agent to ionizing radiation in the presence of oxygen presumably reflects the hydrophobic protein with bound lipids that is required for infectivity (*60*). These data do not eliminate the possibility that the agent also contains a nucleic acid.

Molecular Size of the Scrapie Agent

The extreme resistance of the scrapie agent to inactivation by ionizing radiation raised the possibility that the agent is quite small (*100*). Target calculations have given minimum molecular weights ranging from 64,000 to 150,000 (*82*, *100*). However, two important factors could not be taken into account in these calculations. The first is the possibility that multiple copies of the agent might exist within a single infectious particle as would occur with aggregation. We have good evidence that the agent readily associates with cellular elements and probably aggregates with itself in purified preparations (*47*, *57*, *58*). The second is the efficiency of the cellular repair processes. For example, polyoma virus dsDNA (3×10^6 daltons) has been found to be almost as resistant to ionizing radiation as either viroids or the scrapie agent (*101*). The extreme efficiency of the cellular repair processes for the polyoma virus dsDNA genome accounts for its apparent resistance to damage by ionizing radiation (*82*).

Studies on the scrapie agent in murine spleen have shown a continuum of sizes ranging from 40*S* or less to more than 500*S* by rate-zonal sucrose gradients (*47*, *57*). Parvoviruses are among the smallest viruses identified and they have sedimentation coefficients of 100*S* to 110*S* (*83*). The scrapie agent in preparations extracted with sodium deoxycholate associated with cellular elements when heated t form large infectious particles

Table 4. Properties of the scrapie agent.

Stable at 90°C for 30 minutes
Low molecular weight infectious particles (minimum estimate, 50,000 daltons or less)
Hydrophobic protein (or proteins) is required for infectivity
Resistant to ribonucleases and deoxyribonucleases
Resistant to UV irradiation at 254 nm
Resistant to psoralen photoadduct formation
Resistant to Zn^{2+} catalyzed hydrolysis
Resistant to NH_2OH chemical modification

of >10,000*S* (*47*, *58*). Such particles are the size of mitochondria. Sedimentation studies of CJD agents adapted to both guinea pigs and mice suggest that the sizes of these agents are similar to that observed for the scrapie agent (*73*).

Gel electrophoresis has also shown that the scrapie agent exists as a succession of particles of varying size (*52*, *59*). Sarkosyl agarose gel electrophoresis of partially purified fractions showed that some forms migrated more slowly than DNA restriction endonuclease fragments of 15×10^6 daltons. Some smaller forms of the agent migrated ahead of 3×10^5 dalton DNA fragments. Digestion of crude preparations with nucleases and proteases facilitated the entry of the agent into these gels. One report showed that most of the scrapie agent migrated with 5*S* RNA molecules in the presence of SDS (*51*). We were unable to confirm these findings since SDS inactivated the agent (*24*, *52*).

Until recently, gel filtration studies with anionic detergents and chaotropic ions have given results similar to those described for rate-zonal sucrose gradients and gel electrophoresis. Typically most of the agent eluted in the void volume followed by a continuum of particles apparently of decreasing size (*59*, *67*). In contrast, incubation of the scrapie agent overnight with 10 percent (weight to volume) sulfobetaine 3-14, a zwitterionic detergent, appears to have dissociated the agent (Fig. 3) (*70*). Under these conditions the scrapie agent eluted as a peak behind bovine serum albumin (BSA), but slightly ahead of ovalbumin. If the agent has a globular shape in sulfobetaine 3-14, then it may have a molecular size of 50,000 daltons or less. How much detergent is bound to the agent and how the detergent influences the apparent molecular weight of the agent remains to be determined (*102*). Similar observations have been recorded with another detergent, 1-dodecyl propanediol-3-phosphorylcholine, which is a synthetic derivative of lysolecithin. Confirmation of these findings by rate-zonal sucrose gradient centrifugation is awaited since anomalous behavior of proteins during gel filtration is well known (*103*). Thus, the monomeric form of the scrapie agent may indeed be considerably smaller than that of a viroid, which until now has been the smallest infectious agent known.

If the scrapie agent does have a molecular weight of 50,000 or less, then a nucleic acid within such a globular structure will be too small to code for a protein. A spherical scrapie agent of molecular weight 50,000 would have a diameter of 4 to 6 nm (*104*). Let us assume that the agent has a protective protein which is 1 nm (10 Å) thick. The volume of the core will be 14.1 nm^3. From measurements of DNA packing in crystals and bacteriophage (*105*), there is space for a 12-nucleotide polymer consisting of six base pairs. Dehydration of the polymer would permit 32 nucleotides to be encapsidated. Indeed, if such oligonucleotides exist within the agent, they must have a function other than that of a template directing the synthesis of scrapie coat proteins.

Novel Properties of the Scrapie Agent

The foregoing summary of experimental data indicates that the molecular properties of the scrapie agent differ from those of viruses, viroids, and plasmids (Table 4). Its resistance to procedures that attack nucleic acids, its resistance to inactivation by heat, and its apparent small size all suggest that the scrapie agent is a novel infectious entity. Because the dominant characteristics of the scrapie agent resemble those of a protein, an acronym is introduced to emphasize this feature. In place of such terms as "unconventional virus" or "unusual slow virus-like agent," the term "prion" (pronounced *pree-on*) is suggested. Prions are small *pro*teinaceous *i*nfectious particles which are resistant to inactivation by most procedures that modify nucleic acids. The term "prion" underscores the requirement of a protein for infection; current knowledge does not allow exclusion of a small nucleic acid within the interior of the particle.

Our data and that of other investigators suggest two possible models for the scrapie agent: (i) a small nucleic acid surrounded by a tightly packed protein coat or (ii) a protein devoid of nucleic acid, that is, an infectious protein. While the first model might seem the most plausible, there is no evidence for a nucleic acid within the agent. The sec-

ond model is consistent with the experimental data but is clearly heretical. Skepticism f the second model is certainly justified. Only purification of the scrapie agent to homogeneity and determination of its chemical structure will allow a rigorous conclusion as t which of these two models is correct.

There seems t be little advantage in championing one model over another; however, several previously postulated structures for the scrapie agent can now be discarded. The requirement of a protein for infectivity eliminates the possibilities that the scrapie agent is composed entirely of polysaccharide or nucleic acid. Thus, the replicating polysaccharide and naked nucleic acid–viroid hypotheses are no longer viable. The hypothetical nucleic acid surrounded by a polysaccharide coat can also be eliminated. Studies demonstrating the small size of the scrapie agent clearly distinguish it from conventional viruses, spiroplasma-like organisms, and parasites such as sarcosporidia.

Rigid categorization of the scrapie agent at this time would be premature. Determination of its molecular structure will be required prior to deciding whether prions represent a distinct subgroup of extraordinarily small viruses or a completely different type of pathogen which lacks a nucleic acid genome.

How Do Prions Replicate?

One of the fascinating questions about pri ns concerns their mode of replication. If prions do not contain a nucleic acid genome, then studies on the replication of prions may reveal unprecedented mechanisms of reproduction.

The first possibility is that prions contain a protected nucleic acid and that, like a viral genome, it codes for the protein shell (Table 5). The hypothetical prion genome could derive its protection from the protein-lipid coat or from an unusual chemical structure. Such an unusual genomic structure might confer upon prions the characteristics of proteinaceous particles that are resistant to most procedures that attack nucleic acids.

Alternatively, prions may contain an oligonucleotide that acts as a regulatory element instead of a coding template. This oligonucleotide might act as an inducer t promote the synthesis of prions. Small nuclear RNA's are thought t be regulatory elements controlling the splicing of genes (*106*). If the postulated nucleic acid within the scrapie agent does not code for the protein (or proteins) in its coat, then this would be a major feature distinguishing prions from viruses.

Table 5. Possible mechanisms of prion replication.

Prions contain undetected nucleic acids
Code for prion protein (or proteins)
Activate transcription of host genes coding for prion protein
Prions are devoid of nucleic acids
Activate transcription of host genes coding for prion protein
Code for their own replication by
Reverse translation
Protein-directed protein synthesis

The second possibility is that prions are, in fact, devoid of nucleic acid. If this is the case, then alternative modes of replication for these infectious proteins must exist (Table 5). The macromolecular information required for the synthesis of prions must be contained either in the host cell or in the prion itself.

If cellular genes coding for the scrapie prion do exist, then they are highly regulated, not readily activated, and present in various mammalian cells ranging from mice to monkeys. It is pertinent that hundreds of mice and hamsters inoculated with homogenates from the brains of control animals have never developed a neurological disorder (*9*, *107*). These animals have been observed for up to 1 year, a period of time sufficient to detect one infectious unit in the inoculum. An occasional activation of such cellular genes might explain the sporadic occurrence of CJD with an incidence of $1/10^6$ (*11*). A few clusters of CJD with higher rates of incidence have been identified, and 10 percent of CJD cases are familial. The molecular mechanism by which prions might activate cellular genes which code for their biosynthesis is unknown. The emerging story of oncogenes within retroviruses and their cellular counterparts provides an interesting analogy (*108*).

In addition, we must account for the evolutionary pressure that preserves such hypothetical cellular genes that code for prions. Perhaps these hypothetical genes code for some necessary, related protein or proteins when they are under normal regulation. It may be that tolerance to a normal, cross-reacting gene product might allow the scrapie prion to replicate unnoticed by the immune system. Another explanation for tolerance t ward the scrapie agent involves selective suppression of small populations of potentially reactive lymphocytes (*109*).

Alternatively, prions could code f r their own biosynthesis. This hypothesis contradicts the "c ntral dogma" of molecular biology (*110*). Unorthodox mechanisms such as reverse translation or protein-directed protein synthesis would allow prions to replicate (*111*). We have no precedents for either of these synthetic processes in biology. The possibility that prions are devoid of nucleic acid should be compared to early studies on crystalline tobacco mosaic virus where no RNA was found, and Stanley suggested that the protein of tobacco mosaic virus was autocatalytic (*112*).

Relevant to the mechanism by which the scrapie agent replicates are two observations. First, the various strains of the scrapie and CJD agents have been identified by repeated passage at limiting dilution and by their host range in experimental animals (*45*, *113*, *114*). Second, adaptation of the agent has been observed upon repeated passage in the same host species as evidenced by a reduction in the length of the incubation period (*21*, *113*, *115*). Hadlow observed that the scrapie agent when passaged in mink retains its ability to infect goats, but loses its ability to infect mice (*116*). The agent causing mink encephalopathy has a similar host range (*20*). While adaptation is most readily explained by modification of a nucleic acid genome within the agent, multiple host genes coding for several agents could also explain these observations. The presence of multiple genes coding for different proteins with the same biological activities is emphasized by the occurrence of differing interferons (*117*).

The genetics of the host clearly influences the length of the incubation period for scrapie. Dickinson *et al.* have identified in mice two genetic loci that influence the length of the incubation period (*23*, *118*). In a survey of immunodeficient mice, we found that NZB and NZB × W F_1 mice inoculated intracerebrally have an incubation period of similar magnitude to that found in BALB/c and C57/B1 mice (*61*). In contrast, NZW mice have a significantly shorter incubation period. Further studies with F_2 backcrosses are required to determine if a single gene is responsible for these differences. From these studies and those on the murine CJD agent, we conclude that longer incubation time alleles are autosomal dominant. Murine CJD studies have shown that the D subregion f the H-2 complex plays a central role in controlling the length f th incubation period (*119*). The q allele in this subregion resulted in shorter incubati n times whil the d allele resulted in longer ones.

Conclusion

The consequences of understanding the structure, function, and replication of prions are significant. If prions d not contain a nucleic acid genome which codes for its protein (or proteins), alternative mechanisms of replication and information transfer must then be entertained.

A knowledge of the molecular structure of prions may help identify the etiologies of some chronic degenerative diseases of humans. Development of sensitive probes for detecting prions in such diseases is needed. Diseases where prions might play an etiological role include Alzheimer's senile dementia, multiple sclerosis, Parkinson's disease, amyotrophic lateral sclerosis, diabetes mellitus, rheumatoid arthritis, and lupus erythematosus, as well as a variety of neoplastic disorders (*12*).

The importance of prion research in the potential elucidation of a wide variety of medical illnesses underscores the need for purification of the scrapie agent to homogeneity and the subsequent identification of its macromolecular components. Only then can we determine with certainty whether or not prions are devoid of nucleic acids. Indeed, recent progress in scrapie research has transformed an intriguing yet forbidding prob l m into an exciting and productive area f investigation.

References and Notes

1. S. Stockman, *J. Comp. Pathol. Ther.* **26**, 317 (1913).
2. W. S. Gordon, *Vet. Rec.* **58**, 516 (1946).
3. B. Sigurdsson, *Br. Vet. J.* **110**, 341 (1954).
4. W. J. Hadlow, *Lancet* **1959–II**, 289 (1959).
5. D. C. Gajdusek, C. J. Gibbs, Jr., M. Alpers, *Nature (London)* **209**, 794 (1966).
6. C. J. Gibbs, Jr., *et al.*, *Science* **161**, 388 (1968).
7. C. J. Gibbs, Jr., and D. C. Gajdusek, *ibid.* **182**, 67 (1973).
8. W. J. Hadlow, S. B. Prusiner R. C. Kennedy, R. E. Race, *Ann. Neurol.* **8**, 628 (1980).
9. W. J. Hadlow and S. B. Prusiner, unpublished observations.
10. C. J. Gibbs, Jr., and D. C. Gajdusek, *Nature (London)* **236**, 73 (1972).
11. C. L. Masters, J. O. Harris, D. C. Gajdusek, C. J. Gibbs, Jr., C. Bernoulli, D. M. Asher, *Ann. Neurol.* **5**, 177 (1979).
12. D. C. Gajdusek, *Science* **197**, 943 (1977).
13. M. P. Alpers, in *Slow Transmissible Diseases of the Nervous System*, S. B. Prusiner and W. J. Hadlow, Eds. (Academic Press, New York, 1979), vol. 1, p. 67.
14. C. J. Gibbs, Jr., H. L. Amyx, A. Bacote, C. L. Masters, D. C. Gajdusek, *J. Infect. Dis.* **142**, 205 (1980).
15. I. H. Pattison and G. C. Millson, *J. Comp. Pathol. Ther.* **71**, 171 (1961); I. H. Pattison, M. N. Hoare, J. N. Jebbett, W. A. Watson, *Vet. Rec.* **90**, 465 (1972).
16. S. B. Prusiner and S. P. Cochran, in preparation.
17. D. R. Wilson, R. D. Anderson, W. Smith, *J. Comp. Pathol.* **60**, 267 (1950); W. S. Gordon and I. H. Pattison, *Vet. Rec.* **69**, 1444 (1957); I. H. Pattison and G. C. Millson, *J. Comp. Pathol.* **71**, 350 (1961).
18. R. L. Chandler, *Lancet* **1961–I**, 1378 (1961).
19. S. B. Prusiner, W. J. Hadlow, C. M. Eklund, R. E. Race, S. P. Cochran, *Biochemistry* **17**, 4987 (1978).
20. R. F. Marsh and R. H. Kimberlin, *J. Infect. Dis.* **131**, 104 (1975).
21. R. Kimberlin and C. Walker, *J. Gen. Virol.* **39**, 295 (1977).
22. G. D. Hunter, G. C. Millson, R. L. Chandler, *Res. Vet. Sci.* **4**, 543 (1963); A. G. Dickinson, V. M. Meikle, H. Fraser, *J. Comp. Pathol.* **79**, 15 (1969); A. G. Dickinson and H. Fraser, *ibid.*, p. 363; R. H. Kimberlin and C. A. Walker, *ibid.* **89**, 551 (1979).
23. A. G. Dickinson, V. Meikle, H. Fraser, *J. Comp. Pathol.* **78**, 293 (1968).
24. S. B. Prusiner, D. F. Groth, S. P. Cochran, F. R. Masiarz, M. P. McKinley, H. M. Martinez, *Biochemistry* **19**, 4883 (1980).
25. S. B. Prusiner, S. P. Cochran, D. F. Groth, D. E. Downey, K. A. Bowman, H. M. Martinez, *Ann. Neurol.*, in press.
26. C. M. Eklund, R. C. Kennedy, W. J. Hadlow, *J. Infect. Dis.* **117**, 15 (1967); R. H. Kimberlin, *Sci. Prog. (London)* **63**, 461 (1976); S. B. Prusiner *et al.*, in *Neurochemistry and Clinical Neurology*, L. Battistin, G. Hashim, A. Lajtha, Eds. (Liss, New York, 1980), p. 73; R. N. Hogan, J. R. Baringer, S. B. Prusiner, *Lab. Invest.* **44**, 34 (1981).
27. J. R. Baringer, K. A. Bowman, S. B. Prusiner, *J. Exp. Neurol. Neuropathol.* **40**, 329 (1981).
28. I. Zlotnik, *Acta Neuropathol. Suppl.* **1**, 61 (1962); E. Beck, P. M. Daniel, H. B. Parry, *Brain* **87**, 153 (1964); W. J. Hadlow, R. C. Kennedy, R. E. Race, C. M. Eklund, *Vet. Pathol.* **17**, 187 (1980).
29. J. P. M'Gowan, *Investigation into the Disease of Sheep Called "Scrapie"* (Blackwood, Edinburgh, 1914); J. M'Fadyean, *J. Comp. Pathol.* **31**, 102 (1918).
30. J. Cuille and P. L. Chelle, *C. R. Acad. Sci.* **208**, 1058 (1939); D. R. Wilson, R. D. Anderson, W. Smith, *J. Comp. Pathol.* **60**, 267 (1950); C. M. Eklund, W. J. Hadlow, R. C. Kennedy, *Proc. Soc. Exp. Biol. Med.* **112**, 974 (1963); H. J. Cho, *Nature (London)* **262**, 411 (1976).
31. R. H. Kimberlin and G. D. Hunter, *J. Gen. Virol.* **1**, 115 (1967).
32. J. S. Griffith, *Nature (London)* **215**, 1043 (1967); I. H. Pattison and K. M. Jones, *Vet. Rec.* **80**, 1 (1967); P. Lewin, *Lancet* **1972–I**, 748 (1972); *Can. Med. Assoc. J.* **124**, 1436 (1981).
33. R. A. Gibbons and G. D. Hunter, *Nature (London)* **215**, 1041 (1967); G. D. Hunter, R. H. Kimberlin, R. A. Gibbons, *J. Theor. Biol.* **20**, 355 (1968).
34. D. H. Adams and E. J. Field, *Lancet* **1968–II**, 714 (1968); D. H. Adams, *Pathol. Biol.* **18**, 559 (1970).
35. H. B. Parry, *Heredity* **17**, 75 (1962); in *Virus Diseases and the Nervous System*, C. W. M. Whitty, J. T. Hughes, F. O. MacCallum, Eds. (Blackwell, Oxford, 1969), p. 99; C. D. Darlington, in *ibid.*, p. 133.
36. T. O. Diener, *Nature (London)* **235**, 218 (1972); *Ann. Clin. Res.* **5**, 268 (1973).
37. I. H. Pattison, *J. Comp. Pathol.* **75**, 159 (1965); J. T. Stamp, *Br. Med. Bull.* **23**, 133 (1967); G. D. Hunter, *J. Infect. Dis.* **125**, 427 (1972); D. H. Adams, *Biochem. Soc. Trans.* **1**, 1061 (1973); D. C. Gajdusek, in *Human Diseases Caused by Viruses*, H. Rothschild, F. Allison, Jr., C. Howe, Eds. (Oxford Univ. Press, New York, 1978), p. 231; D. C. Gajdusek and C. J. Gibbs, Jr., in *Viruses and Environment*, E. Kurstak and K. Maramorosch, Eds. (Academic Press, New York, 1978), p. 79.
38. R. G. Rohwer and D. C. Gajdusek, in *Search for the Cause of Multiple Sclerosis and Other Chronic Diseases of the Central Nervous System*, A. Boese, Ed. (Verlag Chemie, Weinheim, 1980), p. 333.
39. E. J. Field, *Br. Med. J.* **2**, 564 (1966); *Dtsch. Z. Nervenheilkd.* **192**, 265 (1967).
40. R. Latarjet, B. Muel, D. A. Haig, M. C. Clarke, T. Alper, *Nature (London)* **227**, 1341 (1970).
41. D. H. Adams and E. A. Caspary, *Br. Med. J.* **3**, 1973 (1967); H. K. Narang, *Acta Neuropathol.* **29**, 37 (1974); A. N. Siakotos, D. Raveed, G. Longa, *J. Gen. Virol.* **43**, 417 (1979).
42. F. O. Bastian, *Arch. Pathol. Lab. Med.* **103**, 665 (1979); A. Gray, R. J. Francis, C. L. Scholtz, *Lancet* **1980–II**, 152 (1980); F. O. Bastian, M. N. Hart, P. A. Cancilla, *ibid.* **1981–I**, 660 (1981).
43. G. D. Hunter, R. H. Kimberlin, S. Collis, G. C. Millson, *Ann. Clin. Res.* **5**, 262 (1973); R. A. Somerville, G. C. Millson, G. D. Hunter, *Biochem. Soc. Trans.* **4**, 1112 (1976).
44. R. F. Marsh, T. G. Malone, J. S. Semancik, *Nature (London)* **275**, 146 (1978).
45. R. H. Kimberlin, *Scrapie in the Mouse* (Meadowfield, Durham, England, 1976), pp. 1–77.
46. G. D. Hunter, in *Slow Transmissible Diseases of the Nervous System*, S. B. Prusiner and W. J. Hadlow, Eds. (Academic Press, New York, 1979), vol. 2, p. 365.
47. S. B. Prusiner *et al.*, in *ibid.*, p. 425.
48. G. C. Millson, G. D. Hunter, R. H. Kimberlin, in *Slow Virus Diseases of Animals and Man*, R. H. Kimberlin, Ed. (Elsevier, New York, 1976), p. 243.
49. J. S. Semancik, R. F. Marsh, J. L. Gealca, R. P. Hanson, *J. Virol.* **18**, 693 (1976).
50. T. G. Malone, R. F. Marsh, R. P. Hanson, J. S. Semancik, *ibid.* **25**, 933 (1978).
51. ———, *Nature (London)* **278**, 575 (1979).
52. S. B. Prusiner, D. F. Groth, C. Bildstein, F. R. Masiarz, M. P. McKinley, S. P. Cochran, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 2984 (1980).
53. A. N. Siakotos, D. C. Gajdusek, C. J. Gibbs, Jr., R. D. Traub, C. Bucana, *Virology* **70**, 230 (1976).
54. P. Brown, E. M. Green, D. C. Gajdusek, *Proc. Soc. Exp. Biol. Med.* **158**, 513 (1978).
55. S. B. Prusiner, *J. Biol. Chem.* **253**, 916 (1978).
56. ———, W. J. Hadlow, C. M. Eklund, R. E. Race, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 4656 (1977).
57. S. B. Prusiner *et al.*, *Biochemistry* **17**, 4993 (1978).
58. S. B. Prusiner *et al.*, *J. Neurochem.* **35**, 574 (1980).
59. S. B. Prusiner, D. F. Groth, S. P. Cochran, M. P. McKinley, F. R. Masiarz, *Biochemistry* **19**, 4892 (1980).
60. S. B. Prusiner *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6675 (1981).
61. K. Kasper, D. P. Stites, K. A. Bowman, S. B. Prusiner, in preparation.
62. A. M. Scanu, *Biochim. Biophys. Acta* **265**, 471 (1972).
63. G. D. Hunter and G. C. Millson, *J. Comp. Pathol.* **77**, 301 (1967); G. D. Hunter, R. A. Gibbons, R. H. Kimberlin, G. C. Millson, *ibid.* **79**, 101 (1969); H. J. Cho, *Intervirology* **14**, 213 (1980).
64. M. P. McKinley, F. R. Masiarz, S. B. Prusiner, *Science* **214**, 1259 (1981).
65. J. A. Yankeelov, C. D. Mitchell, T. H. Crawford, *J. Am. Chem. Soc.* **90**, 1664 (1968); G. E. Means and R. E. Feeney, *Chemical Modification of Proteins* (Holden-Day, San Francisco, 1971), pp. i–254.
66. J. M. Chirgwin, A. E. Przybyla, R. J. MacDonald, W. J. Rutter, *Biochemistry* **24**, 5294 (1979).
67. S. B. Prusiner, D. F. Groth, M. P. McKinley, S. P. Cochran, K. A. Bowman, K. C. Kasper, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4606 (1981).
68. G. D. Hunter and G. C. Millson, *J. Comp. Pathol.* **77**, 301 (1967).
69. F. Szoka, Jr., and D. Papahadjopoulos, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 4194 (1978).
70. S. B. Prusiner and D. F. Groth, unpublished observations.
71. A. Haase, W. J. Hadlow, S. B. Prusiner, unpublished observations. Calcium phosphate and DEAE-dextran were used to promote the entry of the DNA and RNA, respectively, under conditions similar to those in transfection studies of visna virus nucleic acid [A. Haase, B. L. Traynor, P. E. Ventura, *Virology* **70**, 65 (1976)]. The cultured cells were passaged eight times. Portions were removed at each passage and stored at −70°C. Ten percent weight to volume homogenates were diluted tenfold and inoculated into six weanling mice. The mice were observed weekly for clinical signs of scrapie over the next 18 months; none of the mice developed a neurological disorder.
72. M. T. Borras, D. T. Kingsbury, D. C. Gajdusek, C. J. Gibbs, Jr., *J. Gen. Virol.*, in press.
73. D. T. Kingsbury, in preparation.
74. G. D. Hunter, R. A. Gibbons, R. H. Kimberlin, G. C. Millson, *J. Comp. Pathol.* **79**, 101 (1969).
75. G. R. Stark, W. H. Stein, S. Moore, *J. Biol. Chem.* **235**, 3177 (1960).
76. E. E. Manuelidis, E. J. Gorgacz, L. Manuelidis, *Science* **200**, 1069 (1978).
77. R. H. Kimberlin and C. A. Walker, *J. Gen. Virol.* **51**, 183 (1980).
78. D. A. Haig and M. C. Clarke, in *Slow, Latent, and Temperate Virus Infections*, D. C. Gajdusek, C. J. Gibbs, Jr., M. Alpers, Eds. (U.S. Department of Health, Education and Welfare, Washington, D.C., 1965), vol. 2, p. 215; D. L. Mould, A. McL. Dawson, W. Smith, *Res. Vet. Sci.* **6**, 151 (1965).
79. P. D. Lawley and P. Brookes, *Biochem. J.* **89**, 127 (1963); D. M. Brown, in *Basic Principles in Nucleic Acid Chemistry*, P. O. P. Ts'o, Ed. (Academic Press, New York, 1974), vol. 2, p. 1.
80. J. Vinograd, J. Morris, N. Davidson, W. F. Dove, *Proc. Natl. Acad. Sci. U.S.A.* **49**, 12 (1963).
81. T. Alper, W. A. Cramp, D. A. Haig, M. C. Clarke, *Nature (London)* **214**, 764 (1967).
82. R. Latarjet, in *Slow Transmissible Diseases of the Nervous System*, S. B. Prusiner and W. J.

Hadlow, Eds. (Academic Press, [redacted] York, 1979), vol. 2, p. 387.
83. J. A. Rose, *Compr. Virol.* 3, 1 (1974); F. L. Schaffer and C. E. Schwerdt, *Adv. Virus Res.* 6, 159 (1959).
84. T. O. Diener and W. B. Raymer, *Virology* 37, 351 (1969).
85. T. O. Diener, *Viroids and Viroid Diseases* (Wiley, New York, 1979).
86. H. L. Sänger, K. Ramm, H. Domdey, H. J. Gross, K. Henco, D. Riesner, *FEBS Lett.* 99, 117 (1979).
87. E. Mihalyi, *Application of Proteolytic Enzymes to Protein Structure Studies* (CRC Press, Cleveland, 1972).
88. S. B. Prusiner, J. Cleaver, D. F. Groth, unpublished observations.
89. A. D. McLaren and D. Shugar, *Photochemistry of Proteins and Nucleic Acids* (Pergamon, New York, 1964).
90. J. J. Butzow and G. L. Eichhorn, *Biopolymers* 3, 95 (1964); *Nature (London)* 254, 358 (1975).
91. S. T. Isaacs, C. J. Shen, J. E. Hearst, H. Rapoport, *Biochemistry* 16, 1058 (1977).
92. M. P. McKinley, F. R. Masiarz, J. Hearst, S. B. Prusiner, in preparation.
93. J. E. Hearst and L. Thiry, *Nucleic Acid Res.* 4, 1339 (1977); C. V. Hanson, J. L. Riggs, E. H. Lennette, *J. Gen. Virol.* 40, 345 (1978).
94. C. Hanson, personal communication.
95. D. Crowther and J. L. Melnick, *Virology* 14, 11 (1961).
96. K. Borgert, K. Koschel, H. Taüber, E. Wecker, *J. Virol.* 8, 1 (1971).
97. P. Bornstein and G. Balian, *J. Biol. Chem.* 245, 4854 (1970).
98. R. M. Franklin and E. Wecker, *Nature (London)* 184, 343 (1959); E. Freese, E. Bautz-Freese, E. Bautz, *J. Mol. Biol.* 3, 133 (1961); H. Schuster and H.-G. Wittman, *Virology* 19, 421 (1963); J. H. Phillips and D. M. Brown, *Prog. Nucleic Acid Res. Mol. Biol.* 7, 349 (1967); I. Tessman, *Virology* 35, 330 (1968).
99. T. Alper, D. A. Haig, M. C. Clarke, *J. Gen. Virol.* 41, 503 (1978).
100. _____, *Biochem. Biophys. Res. Commun.* 22, 278 (1966).
101. R. Latarjet, R. Cramer, L. Montagnier, *Virology* 33, 104 (1967); J. S. Semancik, T. J. Morris, L. G. Weathers, *ibid.* 53, 448 (1973).
102. J. A. Reynolds, in *Membrane Receptors*, S. Jacobs and P. Cuatrecasas, Eds. (*Receptors and Recognition*, Series B, vol. 11) (Chapman & Hall, London, 1981), p. 33.
103. P. Andrews, *Methods Biochem. Anal.* 18, 1 (1971); Y. Nozaki, N. M. Schechter, J. A. Reynolds, C. Tanford, *Biochemistry* 15, 3884 (1976).
104. A. C. T. Nozth and A. Rich. *Nature (London)* 191, 1242 (1961); C. Tanford, *Physical Chemistry of Macromolecules* (Wiley, New York, 1961), pp. 317–456.
105. R. Langridge, H. R. Wilson, C. W. Hooper, M. H. F. Wilkins, L. D. Hamilton, *J. Mol. Biol.* 2, 19 (1960); G. Giannoni, F. J. Padden, Jr., H. D. Keith, *Proc. Natl. Acad. Sci. U.S.A.* 62, 964 (1969); W. C. Earnshaw and S. R. Casjens, *Cell* 21, 319 (1980).
106. V. W. Yang, M. R. Lerner, J. A. Steitz, S. J. Flint, *Proc. Natl. Acad. Sci. U.S.A.* 78, 1371 (1981).
107. J. M. K. Mackay, *Nature (London)* 219, 182 (1968); I. H. Pattison, K. M. Jones, J. N. Jebbett, *Res. Vet. Sci.* 12, 30 (1971).
108. J. M. Bishop, *Cell* 23, 5 (1981).
109. W. O. Weigle, in *Autoimmunity: Genetic, Immunologic, Virologic and Clinical Aspects* (Academic Press, New York, 1977), p. 141; B. H. Waksman, *Clin. Exp. Immunol.* 28, 363 (1977); L. F. Qualtiere and P. Meyers, *J. Immunol.* 122, 825 (1979).
110. F. Crick, *Nature (London)* 227, 561 (1970).
111. R. Craig, *J. Theor. Biol.* 88, 757 (1981); H. Kleinkauf and H. Von Dahren, *Curr. Top. Microbiol. Immunol.* 91, 129 (1981).
112. W. M. Stanley, *Science* 81, 644 (1935).
113. C. J. Gibbs, Jr., D. C. Gajdusek, H. Amyx, in *Slow Transmissible Diseases of the Nervous System*, S. B. Prusiner and W. J. Hadlow, Eds. (Academic, New York, 1979), vol. 2, p. 87; E. E. Manuelidis and L. Manuelidis, in *ibid.*, p. 147; J. Tateishi, M. Ohta, M. Koga, Y. Sato, Y. Kuroiwa, *Ann. Neurol.* 5, 581 (1979); D. T. Kingsbury, D. A. Smeltzer, H. L. Amyx, C. J. Gibbs, Jr., D. C. Gajdusek, in preparation.
114. A. G. Dickinson and H. Fraser, in *Slow Virus Infections of the Central Nervous System*, V. ter Meulen and M. Katz, Eds. (Springer-Verlag, New York, 1977), p. 3; R. H. Kimberlin and C. A. Walker, *J. Gen. Virol.* 39, 487 (1978).
115. S. B. Prusiner, S. P. Cochran, D. F. Groth, D. Hadley, H. Martinez, W. Hadlow, in *Aging of the Brain and Dementia*, L. Amaducci, A. N. Davison, P. Antuono, Eds. (Raven, New York, 1980), p. 205.
116. W. J. Hadlow, unpublished observations; R. P. Hanson, R. J. Eckroade, R. F. Marsh, G. M. Zu Rhein, C. L. Kanitz, D. P. Gustafson, *Science* 172, 859 (1971).
117. G. Allen and K. H. Fantes, *Nature (London)* 287, 408 (1980); S. Nagata, N. Mantei, C. Weissmann, *ibid.* 287, 401 (1980); M. Rubinstein *et al.*, *Arch. Biochem. Biophys.* 210, 307 (1981).
118. A. G. Dickinson and H. Fraser, in *Slow Transmissible Diseases of the Nervous System*, S. B. Prusiner and W. J. Hadlow, Eds. (Academic Press, New York, 1979), vol. 1, p. 367.
119. D. T. Kingsbury and J. D. Watson, in preparation.
120. This article is dedicated to Dr. Francis A. Sooy on the occasion of his completing a decade as Chancellor of the University of California, San Francisco. I thank F. Elvin, F. R. Masiarz, L. Gallagher, S. P. Cochran, D. F. Groth, M. P. McKinley, K. A. Bowman, D. E. Downey, N. I. Mock, D. P. Stites, and J. R. Baringer for continuing help in these studies; Drs. R. C. Williams, R. C. Morris, Jr., P. Beadheim, D. Bolton, T. O. Diener, W. J. Hadlow, A. Gordon, H. Fields, B. M. Alberts, T. B. Kornberg, I. F. Diamond, W. J. Rutter, M. Rubinstein, and R. M. Stroud for discussions during the preparation of this manuscript; and Drs. F. Seitz, R. Schmid, L. H. Smith, Jr., and J. R. Krevans for support and encouragement. Supported by NIH research grants NS14069 and AG 02132, NSF grant PCM77-24076, a gift from the R. J. Reynolds Industries, and past funds from the Howard Hughes Medical Institute.

Dominance in Fishes: The Relation Between Environment and Abundance

Bernard Einar Skud

Marine fishery studies are replete with comparisons of environmental factors and population abundance or recruitment, and, recently, correlation matrices have been used to compare responses (coefficients) of different species to the same factor (*1*, *2*). Although the studies have included dominant and subordinate species that interact (*3*, *4*), the responses of these species have not been related specifically to their positions in the dominance hierarchy. The purposes f this articles are to compare the relation of temperature to the catch of Atlantic herring (*Clupea harengus*) and Atlantic mackerel (*Scomber scombrus*), species that have alternated as dominant and subordinate in the pelagic biomass off New England and the Canadian Maritime Provinces, and to relate their response to dominance. The relation also is examined for other species, including the California sardine (*Sardinops sagax caerulea*) and anchovy (*Engraulis mordax*).

To paraphrase Daan (*5*), the dominant species is defined as the more abundant of two species that have a functional relation (interact) and whose densities are maintained at distinctly different levels. He specified that replacement, or a change in dominance, required at l ast a 50 percent reduction in abundance of one stock and a comparable increase in the other and that the change be persistent for a number of years.

Interaction Between Herring and Mackerel

Landings (catch) and other estimates of abundance and biomass of herring and mackerel in the Gulf of St. Lawrenc since 1960 indicated that the species interact and have alternated as the dominant species in the pelagic biomass (*4*, *6*). The evidence was based on 15 years of data and on the results obtained from simulation models. The estimates of abundance were from cohort analyses and recruitment surveys. The conclusions of the investigators (*4*, *6*) were tempered by the constraints of their models and by the need for a longer series of empirical data. Supporting evidence of this interaction is apparent in data from Georges Bank (*7*) and from the North Sea (*8*).

In order to satisfy the need for a long-term empirical series I compared the landings of mackerel and herring from the Gulf of Maine to the Gulf f St. Lawrence from the late 1800's t 1960 (Fig. 1) (*9*, *10*). I assumed that long-term

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Letters

New rules on medicines

SIR, – The most recent edition of the BSAVA Canine Medicine textbook has a table of dosages for 20 cardiac drugs. A few of these are licensed for use in dogs and even if captopril is not, enalapril now is. Fortunately, since the Veterinary Medicines Directorate (VMD) has endorsed Dr Aitken's splendidly lenient interpretation of the cascade in relation to companion animals (*VR*, March 11, p 251) we need no longer worry which are licensed and which are not. We simply make the appropriate clinical choice, the arbiter of 'appropriate' being experts such as the author of the chapter, not the VMD (since they will not have data on some of the more modern and appropriate drugs).

Unfortunately, the VMD's latest guidance notes on the new regulations* are like a defensive weather forecast. Encouragingly, 'no suffering which can be treated without placing consumers at risk is acceptable'. Discouragingly, 'products, whether generics or not, authorised for human use, may only be used in accordance with the cascade when there is no authorised veterinary product', that is, no product 'authorised for the condition and species concerned'. Let's consider the case of verapamil, an unlicensed calcium-channel antagonist listed in the chapter. Does the cascade stop me using it when there is (a) an authorised veterinary calcium-channel antagonist, or (b) an alternative veterinary antidysrhythmic, or (c) an alternative veterinary cardiac drug? Only under interpretation (a) am I legal since there is no licensed veterinary calcium-channel antagonist. The BSAVA textbook states that hydralazine is a vasodilator with proven effectiveness in increasing cardiac output in dogs. So I can use hydralazine – or can I, since enalapril, with a different mode of action, is a licensed veterinary vasodilator?

The most menacing statement in the VMD guidance notes, boxed and in capitals on page 1, emphasises that they are 'only a general guide and must not be treated as a complete authoritative statement of the law on any particular case'. Nor, I believe, would a verbal reassurance on a telephone helpline carry any greater legal clout. In a court, all these phrases, unlike those in a code of practice, would not be interpreted by enlightened colleagues, nor by a blind horse, but by lawyers in pursuit of their literal meaning – what they actually say, not what we hope they allow or what they ought to have said.

The answer to a foolishly drafted law is not an acrobatic interpretation. Where companion animals are concerned, this irrational law has the unintended potential grossly to compromise progress in small animal patient care and it needs to be changed. Those who seek to circumvent it will eventually founder and that legal precedent will shackle us all in our legitimate professional judgements. As chairman of the RCVS board for the certificate in animal welfare, I do not want to see the day when case examples include dogs or cats deprived of reasonable therapeutic choices just because the appropriate drug is unlicensed in the EU, though widely advocated by international veterinary experts. I would find this tragic.

President Clinton has just enacted new legislation (S340) precisely to allow American veterinarians to use human drugs when their professional judgement indicates that they are appropriate (and provided the same active constituent is not already available for the same use in that species). The American Veterinary Medical Association has fought for this right for 10 years: why should we relinquish without a whimper? The definitive solution cannot come from the VMD but only through MPs and MEPs alerted to this major threat to animal welfare. The most constructive objective might be to seek a judicial review, under European law, on the grounds that the burden of this law, as currently worded, is disproportionate to any benefit to be gained. In the case of companion animals, the benefit is zero. If this is the appropriate action, time is scarce since I understand that it must be initiated during the first three months of a new law.

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* The Medicines (Restrictions on the Administration of Veterinary Medicinal Products) Regulations 1994 (SI 1994/2987) – Guidance to the Veterinary Profession. VMD Guidance Note, Amelia 8, March 1995

Docking of puppies' tails

SIR, – The veterinary profession must stand firm against the obscene practice of docking puppies' tails (*VR*, February 25, p 203). Docking should be relegated to the bins of history along with cropping for preventing aural haematoma and other practices such as blood letting, firing, ritual slaughter and, in humans, infundibulation (no professional judgement allowed for human surgeons here, Mr Squires).

It is surprising that after other countries (Sweden, Norway) have banned docking for so many years, the Council for Docked Breeds has not yet produced figures (preferably independently verified) showing that the incidence of tail injuries in those countries has risen to nearly 100 per cent of all dogs in breeds that are traditionally docked; even 50 per cent might be persuasive that docking has a widespread prophylactic effect (a figure I suggested as a minimum in my original article on this subject [*VR*, October 3, 1992, p 301]). Furthermore, not one major point in my article has been refuted although the CDB may disagree with my conclusions. In that article I argued that there was little justification for docking as it was wrong to mutilate an animal for a trivial reason, there were often alternatives to the procedure and, finally, that it is wrong to carry out an operation that was likely to cause pain without anaesthetic.

We should continue to assure the CDB that the vast majority of the profession is against this unnecessary mutilation and concurs with the RCVS that it is unethical.

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Diagnosis of BSE

SIR, – We read with interest the study of Wells and colleagues into the correlation between clinical, epidemiological and neuropathological findings in cases of clinically suspect BSE cattle reported between 1987 and 1989 (*VR*, March 4, p 211). While this research provides a limited insight into possible causes of false positives in the early stages of the BSE epidemic, we feel that more could be learnt by investigations into the far larger number of preclinical cases or false negatives which go undetected.

A recent MAFF report (MAFF 1994), reviewed in *The Veterinary Record* (January 14, p 26), states that about 15 per cent of suspected clinical cases are not confirmed at post mortem examination. It claims that this provides 'confidence that no cases are escaping detection'. We find this complacency hard to accept because not only does it ignore preclinical cases, but also the number of missed cases is a function of the sensitivity of the diagnosis and not its specificity. This sensitivity is largely determined by the farmer's awareness and willingness to report clinical cases as well as the diagnostic skills of MAFF veterinarians: some farmers may be reluctant to report such cases because the income lost by becoming a BSE-affected herd is not compensated for by the money received for individual animals slaughtered.

Due to the long incubation period of BSE, many animals will have been slaughtered for other reasons while the disease was still preclinical. In our opinion, the significance of these preclinical cases to the epidemiology of BSE deserves more attention. In particular it would provide valuable epidemiological information concerning the extent of contamination of cattle feed with BSE. In addition it would help to interpret the results of ongoing MAFF cohort studies comparing calves born to clinical cases with the controls which may have been born to preclinical cases.

We believe that MAFF should [redacted] more emphasis on the development of [redacted] suitable diagnostic test which can be used on live animals. This would reduce the dependence on clinical diagnostic techniques and histopathological examination. It might also provide a more sensitive alternative to the use of mouse inoculation tests to detect the presence of low levels of the BSE prion in bovine tissue.

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Reference

MAFF (1994) Bovine Spongiform Encephalopathy – A progress report, November 1994

Dangerous Dogs Act

SIR, – The main issues raised by Baroness Blatch (*VR*, March 11, p 231) in her defence of the Dangerous Dogs Act as it stands are as follows: To allow Lord Houghton's proposed amendments would 'undermine incentives . . . towards responsible dog ownership', would 'risk the perpetuation of fighting dogs in the UK', and 'by allowing owners extra time to register their dogs, would enable some people to bring new dogs into the country'. She does, however, acknowledge that the Act is working in that the pit bull population is reducing and that 'clearly neutering is having an effect'.

To address these issues: Surely the incentives towards responsible ownership of pit bulls already exist in the practice of neutering, muzzling, leash control and microchipping, and surely *not* in mandatory euthanasia. (Elimination of the pit bull from the UK dog population is just as likely, or otherwise, to be achieved now by mandatory neutering of 'suspect' dogs as by euthanasia.)

Perpetuation of at least a small number of fighting dogs in the UK is, unfortunately, likely to be a fact of life despite any amount of legislation. Quite clearly those who undertake the illegal activity of dog-fighting are not those who would have their dogs neutered, muzzled and microchipped to comply with another law! In any case, the purpose of the Act was not solely to remove fighting dogs, otherwise certain Kennel Club registered breeds would inevitably be included, but to eliminate those types of dog most likely to be dangerous to the (human) public in certain circumstances, through actual or potential temperament problems.

The prospect of allowing new dogs of the proscribed breeds to enter the country is extremely unlikely to be influenced by whether or not there is a mandatory destruction order, or a neutering order in force. Even if there is a permanent ban on the importation of new dogs, despite discretion in 'sentencing' in the courts, it will be clear to anyone who has travelled by ferry via the Channel ports recently that the chances of success in illegally importing any number of (perhaps sedated) dogs of any breed are extremely high. Our quarantine laws would hardly matter to anyone with such serious intent.

I sincerely hope that there will soon be a committee of inquiry into the workings of at least Section One of the Dangerous Dogs Act, since it should surely by now be made clear to the Home Office that the number of successful convictions under the Act would increase, and the public costs involved in defending these cases to the hilt would decrease, if the stigma imposed on the judiciary to order euthanasia of such dogs, without question, was lifted. The new proposals are after all intended to be discretionary rather than mandatory, and in certain cases euthanasia might still be carried out if deemed appropriate, thus not significantly weakening the Act. Also, the fact that Section Three of the Act is filled with loopholes to the disadvantage of the prosecuting authorities, in addition to the other well-voiced arguments, should surely give the Home Office some incentive for discussion.

My own proposal would be temporarily to reopen the Index of Exempted Dogs to allow a voluntary amnesty for those people who believe they already own a dog likely to be subject to Section One of the Act, and to allow dogs already seized under that section to be 'made legal' with their owner's consent, thereby saving considerable court time and expense. Subsequently, I would propose that the index should only be reopened at the discretion of the courts for dogs found to be subject to Section One of the Act as a result of court proceedings, and only at the discretion of the judge in any such case.

I think it would also be necessary in the long term for there to be a system for owners to apply to the court to have dogs voluntarily added to the index should they be unfortunate enough accidentally to breed or own a dog which appeared strongly to resemble a proscribed breed.

Since there are now less than one hundred dogs being held nationwide under Section One of the Act, this would be the time to lobby the Home Office with these proposals, and hopefully rid the dog world, and parts of the veterinary establishment, of the animosity which the Act has engendered over the past three and a half years.

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'Normal' blood copper levels in horses

SIR, – Further to the letter from Suttle and others regarding overestimation of copper deficiency in horses (*VR*, February 4, p 131), the data in Fig 1 are from a survey of reference ranges used in veterinary laboratories worldwide. As the data show, there is wide variation in the 'normal' range currently used for equine serum copper values. The lower limit of the reference range varies between 8 and 21 µmol/litre. Atomic absorption spectrophotometry (AAS) is the most commonly reported analytical method in this survey.

Some published data indicate that 'high' equine blood copper levels are commonly found, particularly in growing and racing thoroughbreds. A mean (sd) blood copper value (AAS) of 21·8 µmol/litre (2·4) was reported by Egan and others (1980) for 329 samples collected from 51 two-year-old Irish thoroughbreds sampled throughout the racing season. More recently, O'Donoghue (1991) reported a mean (sem) plasma copper value (AAS) of 22·3 µmol/litre (0·3) in 201 thoroughbred yearlings on 17 Irish stud farms. Individual values ranged between 14 and 44 µmol/litre.

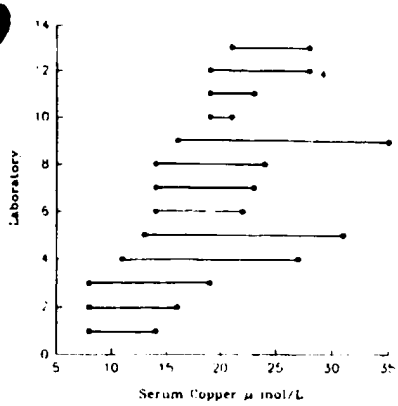

FIG 1: Reference ranges used for equine serum copper concentration in 16 veterinary laboratories (4 - Four laboratories with the same reference range)

These values probably represent the level of concentrate ration feeding and copper supplementation practised on stud farms and racing stables rather than the norm for unsupplemented horses.

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References

EGAN, D. A., CULLEN, J. & McLAUGHLIN, J. G. (1980) *Veterinary Research Communications* **4,** 107

O'DONOGHUE, D. D. (1991) MVM thesis. National University of Ireland

Suspected carbon monoxide poisoning in a dog

SIR, – Further to the letter from Jeremy Hopkins (*VR*, February 25, p 204), I thought the following report might be of interest.

I examined a dog with possible carbon monoxide poisoning last winter in which the major presenting sign was cherry red mucous membranes, particularly the conjunctiva. The dog (a six-year-old male boxer) was also slightly lethargic, tachycardic (heart rate 180/min) and bad tempered. As far as the owner was concerned this dog had 'sore eyes'. The dog had a superficial corneal ulcer on one eye but no ocular discharge or blepharospasm.

On taking the history I was told the dog had been 'lying beside the fire and not been interested all day'. As it had been a gas fire, I advised the owners to get the fire checked and not to use it until it was certified safe. I treated the dog's corneal ulcer with chloramphenicol eye ointment and advised that the dog should be rested at home with plenty of fresh air available.

The Gas Board examined the fire and found three faults, including a restricted flue.

Table 3. Half-life determinations of ^{176}Lu.

Half-life (10^{10} yr)	Method	References
4	β counting, gas counter	(*12*)
7.3 ± 2	β counting with absorbent, gas counter	(*13*)
2.4	β counting, gas counter	(*14*)
2.15 ± 0.1	γ counting, NaI crystal	(*15*)
2.1 ± 0.2	γ counting, NaI crystal	(*16*)
2.8	Proportional β counting, gas counter	(*16*)
2.17 ± 0.35	$^{176}Lu/^{176}Hf$ determination on a dated mineral	(*1*)
3.6 ± 0.1	$\gamma\gamma$ coincidence, NaI crystal	(*9*)
3.2	Proportional β counting with absorbent, gas counter	(*9*)
2.18 ± 0.06	β counting, liquid scintillator	(*17*)
3.5 ± 0.14	γ counting, NaI crystal	(*10*)
3.68 ± 0.06	$\gamma\gamma$ coincidence, NaI crystal	(*10*)
3.56 ± 0.05	β-γ coincidence	(*10*)
5.0 ± 0.3	γ-γ coincidence, NaI crystal	(*11*)
3.3 ± 0.5	$^{176}Lu/^{176}Hf$ determination on two dated minerals	This report

of error; they indicate an age of crystallization of 900 ± 20 million years, in good agreement with ages (880 to 930 million years) of other pegmatitic minerals in the same region (*7*).

The three Pb/U ages on the priorite are also concordant, around 1080 ± 50 million years (*8*).

The values for the half-life of ^{176}Lu deduced from the two minerals agree with each other within their limits of error; thus it may reasonably be admitted that both minerals have behaved as closed systems for Lu and Hf since their crystallization. The weighted average value is $3.3 \pm 0.5 \times 10^{10}$ years. The weight of each determination is inversely proportional to its precision.

The various determinations of the half-life of ^{176}Lu given in Table 3 range from 2 to 7×10^{10} years. The physical determinations of MacNair (*9*) and of Brinkman *et al.* (*10*) are in agreement and seem to be the most reliable. Brinkman *et al.* extracted the radioactive impurities from the Lu and found concordant values close to 3.6×10^{10} years by three counting methods.

Our determination agrees with this value within the limits of error. It definitely differs, however, from a recent value of 5.0×10^{10} years (*11*).

The geological determination of Herr *et al* ($2.17 \pm 0.35 \times 10^{10}$ years) (*1*) is significantly lower. A possible explanation is that their sample did not behave as a closed system either for Lu and Hf or for U and Pb. Losses of lead may be inferred from the admitted age of 810 million years, which is lower than the age of pegmatitic minerals from the same region, and from the discordancy of the apparent Pb/U ages.

Additional Lu-Hf determinations on other dated minerals are necessary to obtain a more precise value for the half-life of ^{176}Lu and to obtain information about the geochemical behavior of these two elements. The analytical and the isotope dilution techniques already developed permit the application of the $^{176}Lu/^{176}Hf$ dating method to 10 g of minerals that are 1000 million years old and that contain 100 parts per million of Lu.

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References and Notes

1. W. Herr, E. Merz, P. Eberhardt, P. Signer, *Z. Naturforsch. A* **13**, 268 (1958).
2. A. Boudin, *Radioactive Dating and Methods of Low-Level Counting* (International Atomic Energy Agency, Vienna, 1967), p. 515; ——— and M. Dehon, *Geochim. Cosmochim. Acta* **33**, 142 (1969).
3. A. Boudin and F. Hanappe, *Radiochim. Acta* **8**, 188 (1967).
4. The first steps in the chemical procedure are borax fusion of the mineral and Hf spike and dissolution of the flux in 6*M* HCl. The procedure for Lu is as follows: addition of Lu spike to a small aliquot of the solution; coprecipitation of rare earths with iron III through the addition of NH_4OH; ether extraction of the iron from the redissolved (6*M* HCl) precipitate; evaporation of solution, dissolution of rare earths in α-hydroxyisobutyric acid [see D. L. Massart and J. Hoste, *Anal. Chim. Acta* **28**, 378 (1963)]; separation of Lu by ion exchange techniques with α-IHBA used as the elutant; elution of Lu in the first 100 ml of α-IHBA; oxidation of α-IHBA by $HClO_4$; redissolution of Lu in 1*N* HCl. The procedure for Hf is as follows: extraction of Hf + Zr from the remaining 6*M* HCl solution by TTA (thenoyltrifluoroacetone) [see F. L. Moore, *Anal. Chem.* **28**, 997 (1956)]; extraction with 4 percent HF; evaporation of HF solution to dryness; Hf transformed to oxychloride by 1*N* HCl.
5. D. L. Massart and J. Hoste, *Anal. Chim. Acta* **42**, 15 (1968).
6. S. Deutsch, D. Ledent, P. Pasteels, Internal Report (Service de Géologie et Géochimie Nucléaires, Université Libre de Bruxelles, Bruxelles, 1965), 168 pp.
7. H. Neuman, *Nor. Geol. Tidsskr.* **40**, 173 (1960); E. Welin and G. Blomqvist, *Geol. Foeren. Stockholm Foerh.* **86**, 33 (1964).
8. D. Ledent, E. Picciotto, G. Poulaert, *Bull. Soc. Belge Geol. Paleontol. Hydrol.* **65**, 233 (1958); P. Eberhardt, J. Geiss, H. R. von Gunten, F. G. Houtermans, P. Signer, *ibid.*, p. 251.
9. A. MacNair, *Phil. Mag.* **6**, 851 (1961).
10. G. A. Brinkman, A. H. W. Aten, J. Th. Veenboer, *Physica* **31**, 1305 (1965).
11. K. Sakamoto, *Nucl. Phys. A* **103**, 134 (1967).
12. M. Heyden and W. Wefelmeyer, *Naturwissenschaften* **24**, 612 (1938).
13. W. F. Libby, *Phys. Rev.* **56**, 21 (1939).
14. A. Flammersfeld and J. Mattauch, *Naturwissenschaften* **31**, 66 (1943); A. Flammersfeld, *Z. Naturforsch. A* **2**, 86 (1947).
15. J. R. Arnold, *Phys. Rev.* **93**, 743 (1954).
16. R. N. Glover and D. E. Watt, *Phil. Mag.* **2**, 699 (1957).
17. D. Donhoffer, *Nucl. Phys.* **50**, 489 (1964).
18. P. Pasteels, private communication.
19. We thank Professor E. Picciotto, who suggested this work. Supported by the Belgian Institut Interuniversitaire des Sciences Nucléaires. One of us (A.B.) received support from the Belgian Fonds National de la Recherche Scientifique.

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Experimental Allergic Encephalomyelitis: Synthesis of Disease-Inducing Site of the Basic Protein

Abstract. *A highly encephalitogenic peptide whose structure resembles the sequence of amino acids surrounding the single tryptophan residue in the encephalitogenic A1 protein from bovine myelin was synthesized. This peptide is similar in the sequence to peptic peptide E and tryptic T27, derived directly from the A1 protein, and is as active on a molar basis as the A1 protein. The major disease-inducing site of the A1 protein resides in a linear sequence of nine amino acids: H-Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Lys-OH. This region of the A1 protein is apparently the major encephalitogenic determinant since specific modification of the tryptophan residue in the A1 protein with 2-hydroxy-5-nitrobenzyl bromide destroyed its encephalitogenic activity.*

The factor in the central nervous system responsible for experimental allergic encephalomyelitis (EAE) is a basic protein (A1 protein) present in myelin where it constitutes at least 30 percent of the total protein (*1*, *2*). At doses of 0.1 μg or greater, the A1 protein induces EAE (*1*) in guinea pigs. The pathogenesis of EAE appears to be associated with an immune response involving sensitized lymphocytes (*3*), presumably by a delayed-type hypersensitive mechanism. Thus, EAE provides a useful model for the study of autoimmune disease and may have relevance to some human demyelinating diseases such as multiple sclerosis.

The A1 protein is a basic protein

and its m lecular weight is 18,000 (*4*). In solution it has an open conformation, as revealed by its high intrinsic viscosity (*4*), and rapid cleavage by proteolytic enzymes (*5*) and resistance t denaturation. Biological activity remains after th protein is heated to 100°C for 1 hour or treated with 8*M* urea (*4*). Optical rotatory dispersion measurements indicate that there is neither an α helix nor a β structure in A1 protein (*6*, *7*). The complete amino acid sequence of the bovine A1 protein has been determined (*8*).

The peptide E (Table 1) used in our study was derived (*9*, *10*) from a pepsin digest of the bovine A1 protein. The structure of peptide E shown in Table 1 differs from that reported (*9*) by the deletion of a glycine and a serine residue, with a total of 14 residues. The correct structure for peptide E was det rmined from chymotryptic and tryptic peptides. After chymotrypsin digestion of the tetradecapeptide, a tripeptide Ser-Arg-Phe (*11*) and a dipeptide Ser-Trp were isolated by high-voltage electrophoresis; the Phe-Ser-Trp sequence is established by this and previous data (*9*). From the COOH-terminal end, a small quantity (20 percent) of the peptide Pro-Gly-Phe was obtained after digestion with trypsin. The COOH-terminal phenylalanine was determined by hydrazinolysis of the intact peptide. Thus, for the first nine residues the amino acid sequence of peptide E is identical with that of peptide T27 obtained from the tryptic digest (*8*). The sequence of all peptides was determined by the direct Edman degradation procedure (*8*).

The HNB-A1 protein was prepared by treating the A1 protein with 2-hydroxy-5-nitrobenzyl bromide (*12*) as described (*13*). By spectrophotometric analysis of the HNB-A1 protein it was established that one tryptophan residue had reacted per 18,000 daltons (*6*, *13*), a value close to that determined by sedimentation-equilibrium for the molecular weight of the A1 protein.

The synthetic peptides used in our study are shown in Table 1; all synthetic peptides were made by the Merrifield solid-phase procedure (*14*) with the use of (*11*) *t*-BOC-CBZ-Lys, *t*-BOC-*O*-Bz-Glu, *t*-BOC-*O*-Bz-Ser, *t*-BOC-NO_2-Arg, *t*-BOC-Gly, *t*-BOC-Ala, *t*-BOC-Trp, *t*-BOC-Phe, and *t*-BOC-PNP-Gln, and chloromethylated copolystyrene cross-linked with 2 percent divinylbenzene. The *t*-BOC protecting groups were removed with a solution of trifluoroacetic acid, methylene chloride, and mercaptoethanol (50 : 45 : 5). The peptides were neutralized with 12 percent triethylamine in chloroform. The peptide bonds, except when glutamine was added, were formed with *N*,*N*-dicyclohexylcarbodiimide in methylene chloride; bonds with *t*-BOC-nitro-L-Arg and *t*-BOC-Trp were formed in dimethylformamide and methylene chloride (1:2). Glutamine was coupled by use of the active ester *t*-BOC-Gln-PNP in dimethylformamide and acetic acid (100:1). The protecting groups were removed from the peptides, and the peptides were removed from the resin by treating 1 g of resin-peptide complex with 5 ml of HF and 0.5 ml of anisole at 0°C for 0.5 hour (*15*).

The synthetic material was then purified by gel filtration in 0.5*N* acetic acid on a layered column of Sephadex G-10 and G-25 (4 by 100 cm). In each case the elution pattern, determined by the optical density at 280 nm, contained a single main peak which eluted prior to,

Table 1. Sequences of synthetic and derived peptides.

Peptide	Sequence
	Synthesis
S1	Ser-Arg-Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Lys
S2	Ser-Arg-Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln
S3	Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln
S4	Ser-Arg-Phe-Ser-Trp-Gly-Ala-Glu-Gly
S5	Ser-Arg-Phe-Ser-Trp-Gly-Ala
S6	Ser-Arg-Phe-Ser-Trp
S7	Phe-Ser-Trp-Gly-Ala
S8	Ser-Arg-Phe-Gly-Ser-Trp
S9	Ser-Arg-Phe-Gly-Ser-Trp-Gly-Ala
S10	Ser-Arg-Phe-Gly-Ser-Trp-Gly-Ala-Glu-Gly-Gln
	Peptic digest
E	Ser-Arg-Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Lys-Pro-Gly-Phe
	Tryptic digest
T27	Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Lys

Table 2. Biological activities of peptides and modified A1 proteins.

Material*	Dose (μg)	No. of animals showing EAE/No. of animals tested†		Skin test‡
		Clinical	Clinical + histologic	
Peptide S1	0.33	1/4	2/4	Negative
	3.3	2/4	3/4	
	5.0	3/4		
	33.0	2/4	3/4	
	50.0	2/4		
Peptide S2	3.3	0/4	0/4	Negative
	33	0/4	0/4	
Peptide S3	3.3	0/4	0/4	Negative
	33	0/4	0/4	
Peptide T27	0.33	1/4	4/4	
	3.3	4/8	7/8	Negative
	10	4/4	4/4	
Peptide E	0.33	3/4	3/4	Negative
	3.3	2/4	4/4	
	10	3/4	3/4	
A1 protein (bovine)	3.3	0/4	2/4	Positive (19 mm)
	50	4/5	5/5	
HNB-A1 protein	3.3	0/5	0/5	Positive (17 mm)
	20	0/5	0/5	

* Other peptides which were tested at 5 and 50 μg include peptides S9, S4, S5, S6, S7, S8, and S10. None of these peptides was encephalitogenic in guinea pigs, as judged by clinical and histologic criteria, and none gave a positive skin test. † In general, the appearance of clinical signs of EAE occurred between 12 and 16 days after injection. Occasionally, signs were observed earlier or later than this period. The peptides showed the same time sequence of the A1 protein. Guinea pigs were sacrificed at 20 days for histologic examination of brain and spinal cord. The criteria for histologic lesions have been described (*1*). ‡ The skin test was carried out 8 to 9 days after injection of 33 μg of A1 protein in the usual way to induce EAE. Samples were tested from 0.1 to 50 μg in 0.05 ml of saline. Maximum response occurred after 24 hours. Positive reactions consisted of an area of erythema greater than 8 mm. The average diameters found with 20 μg of A1 protein and HNB-A1 protein were 19 and 17 mm, respectively.

and separated from, various lesser quantities of smaller peptides. High-voltage electroph resis at *p*H 4.6 showed that this main peak was homogeneous; the trailing material often contained two to four peptides that migrated differently from the maj r peptide. Based on the relative areas of the major and minor peptide fractions, the yield varied from 60 to 90 percent. Approximately 200 to 400 mg of purified peptide was usually obtained. The amino acid analyses of the hydrolyzates of the synthetic peptides, determined with a Beckman amino acid analyzer (*9*), were consistent with the formulations given in Table 1; the residues were found in integral molar ratios within ± 5 percent.

The assays (*1*) for encephalitogenic activity of the peptides (Table 2) show that nly peptide S1 am ng the synthetic peptides is encephalitogenic. Similar clinical signs were ev ked by the synthetic peptide S1, peptides E and T27, and the A1 protein. The histological changes (Fig. 1) for peptide S1 included increased vascular permeability, cellular infiltration, and perivascular cuffing. The predominant cell type was mononuclear, consisting of lymphocytes and histiocytes; in several lesions a few plasma cells were identified. The lesions were present in the meninges, choroid plexus, and around small blood vessels in the brain and spinal cord. These lesi ns appeared similar t those induced by the A1 protein and whole spinal cord as described (*1*).

Peptide S1, with 11 residues, is id ntical to the peptic peptide E through the lysine residue, and therefore additional residues (the Pro-Gly-Phe sequence) at the COOH-terminal end of peptide E are not required for encephalitogenic activity. Although the EAE assay is only partially quantitative, it appears that peptide S1 and peptid E have similar activities; both produced clinical signs and histologic lesions at 0.33 μg per animal, the lowest dose tested. In the one group of guinea pigs shown in Table 2, peptide E was highly effective clinically at 0.33 μg; the animals exhibited classical hind-leg paralysis, tremors, weight loss, and death (*1*). In the corresponding group tested with 0.33 μg of peptide S1, one animal was paralyzed; the oth rs showed hind-leg weakness, but not a definite paralysis. All of the other synthetic peptides, such as peptide S2, gave no signs of EAE, either clinically or histologically. Peptide S2 differs from S1 only by the absence of COOH-terminal lysine, thus setting the length limit of the encephalitogenic region at the COOH-terminal end.

Thus peptide T27 is highly encephalitogenic, having activity of the same order as synthetic peptides S1 and peptic peptide E. It contains only nine residues and is identical with the other two peptides in the region from the phenylalanine to the lysine residues. Thus, the encepahlitogenic region can be reduced to a sequence of nine residues, the NH_2-terminal phenylalanine to COOH-terminal lysine. The isolation of an encephalitogenic peptide from a tryptic digest of the A1 protein is significant in that it is composed of the same sequence of amino acids as the active peptic polypeptides, namely, that surrounding the singie tryptophan residue. Previously, however, when we examined the tryptic peptide fractions derived from the A1 protein, we found (*5*) a very limited activity; only 20 percent of the animals showed clinical signs. When compared with that of the peptic peptide fractions, the activity of the tryptic peptides appeared borderline (*16*). Apparently, when tested as part of the tryptic peptide fractions, other peptides interfere and mitigate its encephalitogenic activity. These data are consistent with that f Carnegie *et al.*

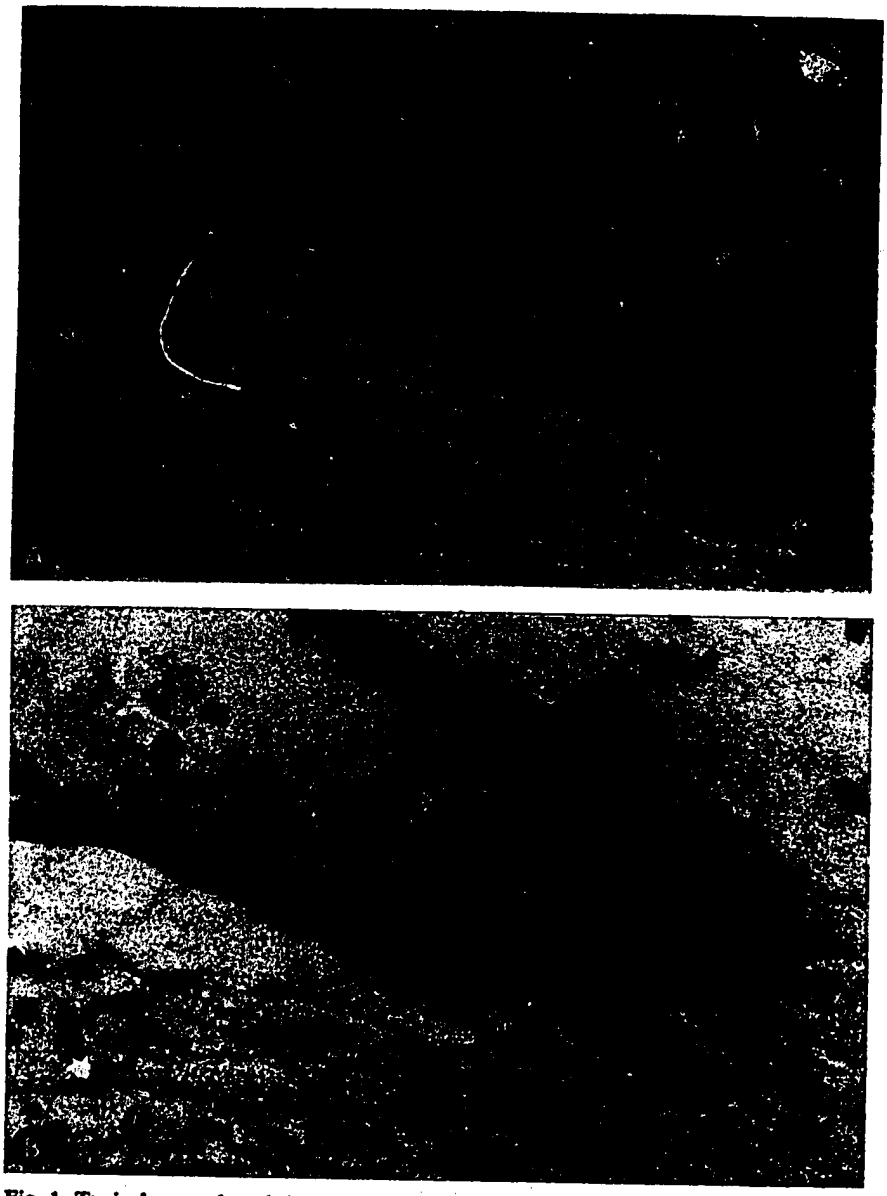

Fig. 1. Typical examples of the perivascular cuffing induced in the cerebral hemispheres by 0.33 μg of synthetic peptide S1 are shown at magnification × 200 for (A) and × 650 for (B). The sections were stained with hematoxylin and eosin.

(*17*) who found that a tryptic peptide mixture derived from human basic protein was encephalitogenic.

Our results reveal that the encephalitogenic activity of the bovine A1 protein is primarily due to a short, linear sequence of amino acids surrounding the single tryptophan residue. The requirements for encephalitogenic activity are precisely determined within a framework of nine amino acids or less which are represented by the common sequence found within peptic peptides E and E1, tryptic peptide T27, and synthetic peptide S1, the only synthetic peptide which was active. This sequence is:

Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Lys

The question arises concerning the number of regions in the A1 molecule which can independently induce EAE. Is the nine-residue tryptophan region the only encephalitogenic determinant and the remaining 95 percent of the molecule superfluous? It appears that th region defined by the amino acid sequence around the tryptophan residue is the major encephalitogenic determinant because (i) specific chemical modification of the tryptophan residue with 2-hydroxy-5-nitrobenzyl bromide greatly reduces the encephalitogenic activity of the A1 protein (Table 2) and (ii) the only encephalitogenic peptides derived from the peptic and tryptic digests of the A1 protein come from the tryptophan region. Kibler *et al.* (*18*) have reported that a peptide of 45 residues, derived from bovine spinal cord, is encephalitogenic in rabbits at doses of 50 μg per animal. We have now derived (*8*) the identical peptide from a peptic digest of the A1 protein; it occupies a portion of the polypeptide chain near the NH_2-terminal region and does not overlap the tryptophan region. Theref re two independent encephalitogenic sites may exist in the A1 protein, and considerable species variability may exist in response to these regions. It is not clear why the bovine peptide of Kibler *et al.* (*18*) is not encephalitogenic in guinea pigs (*19*), whereas the bovine A1 protein is highly encephalitogenic.

The delayed skin test (Table 2) with the encephalitogenic peptides in guinea pigs sensitized with the A1 protein was negative in each case. However, the HNB-A1 protein, which is nonencephalitogenic, nonetheless gives a delayed-type skin reaction equivalent to that of the A1 protein (*1*). Thus, the skin test, which has been correlated with induction f EAE (*1*, *3*), can be differentiated from the disease process; this suggests that more than ne site f the A1 protein molecule may induce the delayed-skin response.

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References and Notes

1. E. H. Eylar, J. Salk, G. Beveridge, L. Brown, *Arch. Biochem. Biophys.* 132, 34 (1969); A. Nakao, W. Davis, E. R. Einstein, *Biochim. Biophys. Acta* 130, 163 (1966).
2. M. W. Kies, *Ann. N.Y. Acad. Sci.* 122, 161 (1965).
3. E. C. Alvord, in *The Central Nervous System*, O. T. Bailey and D. E. Smith, Eds. (Williams & Wilkins, Baltimore, 1968).
4. E. H. Eylar and M. Thompson, *Arch. Biochem. Biophys.* 129, 468 (1969).
5. G. Hashim and E. H. Eylar, *ibid.* 129, 635 (1969).
6. Y. Oshiro and E. H. Eylar, *ibid.*, in press.
7. F. B. Palmer and R. M. C. Dawson, *Biochem. J.* 111, 629 (1969).
8. E. H. Eylar, F. Westall, J. Caccam, G. Hashim, in preparation.
9. E. H. Eylar and G. Hashim, *Proc. Nat. Acad. Sci. U.S.* 61, 644 (1968).
10. G. Hashim and E. H. Eylar, *Arch. Biochem. Biophys.* 129, 645 (1969).
11. Abbreviations for residues: Pro, proline; Ser, serine; Arg, arginine; Phe, phenylalanine; Trp, tryptophan; Lys, lysine; Glu, glutamic acid; Gly, glycine; Glu, glutamine; and Pro, proline. Other abbreviations are: HNB, 2-hydroxy-5-nitrobenzyl bromide; BOC, butoxycarbonyl; CBZ, carbobenzoxy; Bz, benzyl; PNP, *p*-nitrophenyl.
12. T. Barman and D. Koshland, *J. Biol. Chem.* 242, 5771 (1967).
13. E. H. Eylar and G. Hashim, *Arch. Biochem. Biophys.* 131, 215 (1969).
14. R. B. Merrifield, *J. Amer. Chem. Soc.* 85, 2149 (1963); G. R. Marshall and R. B. Merrifield, *Biochemistry* 4, 2394 (1965); A. Marglin and R. B. Merrifield, *J. Amer. Chem. Soc.* 88, 5051 (1966).
15. S. Sakakibara, Y. Shimonishi, Y. Kishida, M. Okada, H. Sugihora, *Bull. Chem. Soc. Japan* 40, 2164 (1967); J. Lenard and A. B. Robinson, *J. Amer. Chem. Soc.* 89, 181 (1967).
16. M. Kies, E. B. Thompson, E. C. Alvord, *Ann. N.Y. Acad. Sci.* 122, 148 (1965).
17. P. Carnegie, B. Bencina, G. Lamoureux, *J. Biochem.* 105, 559 (1967).
18. R. Kibler, R. Shapira, S. McKneally, S. Jenkins, P. Selden, F. Chow, *Science* 164, 577 (1969).
19. M. Kies, personal communication.
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Photochemical Oxidants: Effect on Starch Hydrolysis in Leaves

Abstract. *Starch-filled leaves of plants which have been subjected to low dosages of naturally occurring photochemical oxidants, ozone, or peroxyacetyl nitrate hydrolyze their starch more slowly when placed in the dark. Delayed hydrolysis occurs irrespective of whether the oxidants were applied during the light or dark period. Occasionally this effect is evident only in the interveinal areas.*

In conjunction with research on tobacco mosaic virus (TMV) at Arcadia, California, P. C. Cheo found that starch in normal appearing, inoculated cucumber cotyledons failed to disappear as it had from those similarly treated in studies conducted at Wenatchee, Washington. The formation of starch lesions is a critical determining feature in the TMV assay (*1*). In this assay the virus concentration is proportional to the number of leaf spots which fail to translocate starch due to interference by the infecting virus. Failure of starch hydrolysis in the uninfected leaf portions made the assay useless. Because we suspected that air pollution (photochemical oxidants) was causing this problem, we installed activated carbon filters in the greenhouse. Starch disappearance, as expected, then occurred after the cotyledons were held in the dark. We conducted further studies into the effect of photochemical air pollutants on starch hydrolysis in leaves.

We observed that starch retention occurs over the entire leaf blade when plants are exposed to low dosages of naturally occurring airborne oxidants such as ozone or peroxyacetyl nitrate (PAN). Starch normally accumulates in plant leaves during the daylight hours when photosynthesis exceeds the rate of translocation of products of photosynthesis from the leaves. The following night this starch undergoes hydrolysis and is exported to areas of growth and storage. Photochemical oxidants somehow block or retard certain steps of the starch hydrolysis–translocation process.

Seeds of cucumber *Cucumis sativa*, bean *Phaseolus vulgaris* cv. "Pinto," *Cassia occidentalis*, and *Mimulus cardinalis* were grown in greenhouses equipped with activated carbon air filters to remove atmospheric oxidants. The 3-week-old seedlings were then exposed for varying lengths of time to